PATHOGENESIS OF CRIMEAN-CONGO HAEMORRHAGIC FEVER (CCHF) – INTERACTION OF IMMUNE RESPONSE, VIRAL LOAD AND CLINICAL COURSE

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Pathogenesis of Crimean-Congo haemorrhagic fever (CCHF) - interaction of immune response, viral load and clinical course

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Abstract

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Introduction

Crimean-Congo haemorrhagic fever a potentially fatal acute viral disease that is geographically widespread across southern areas of Africa and Eastern Europe, Russia and the Middle East. It is a major emerging infectious diseases threat, but limited data exist on the clinical course, host immune response, viral dynamics and associated coagulopathy.

Aims

A cohort of adults with PCR positive CCHF in Turkey was recruited to determine the viral load dynamics and coagulopathy over the course of clinical infection and to investigate the host immune response through serial measurement of cytokine/chemokine levels and quantitative serological response, related to disease severity and clinical outcomes.

Methods

Prospective observational cohort study. Demographic, clinical and laboratory data were collected from patients admitted with confirmed CCHF in Turkey, between May 2014 and August 2015. Diagnosis was confirmed on admission by a positive CCHF polymerase chain reaction (PCR) or IgM enzyme-linked immunosorbent assay. Quantitative RT-PCR, serology, luminex flow cytometry and rotational thromboelastometry (ROTEM) was undertaken at admission, during acute illness and in convalescence.

Results

During the study period 104/144 patients recruited were confirmed to have CCHF. The majority were male (66/104) and had mild disease (63/104), with a median age of 50 years. Median temperature at admission was 37.5^0C with fever, headache, myalgia, lethargy and vomiting the most frequent symptoms reported. 23% of participants had an episode of bleeding and 37% received blood product transfusion. Higher CCHF viral load at admission was associated with increased disease severity (p=0.02), lower platelet counts (p=0.02) and fatal outcome (p=0.01). APTT, PT, LDH, CK and creatinine correlated with CCHF viral load. Median clearance of CCHFV from plasma was 5 days, occurring more rapidly in mild disease. Urine is rarely PCR positive in acute infection (8/578 samples) or in convalescence (0/101 samples).

At admission 43/102 (42.2%) participants had a positive CCHFV IgM and 51/102 (50.0%) had a positive CCHFV IgG. 13/61 (21%) participants remained IgM positive at 1 year after infection. IL-10, IP-10, GM-CSF, IL-12, IL-17A and IL-8 were significantly elevated in moderate/severe cases, with IL-10, IL-6, IL-8, TNF-α and MCP-1 higher in fatal cases. TNF-α, IL-6, IFN-A2, IL-15, IL-8 and MIP-1β correlated with CCHF viral load. At admission, EXTEM S clotting time (CT), amplitude 10 minutes (A10), clot formation time (CFT) and maximum clot firmness (MCF) were significantly different between mild and moderate/severe cases. EXTEM S MCF and A10 were lowest on days 4-6 of illness. There were no significant differences in FIBTEM A10/MCF by severity and no hyperfibrinolysis.

Conclusions

CCHF frequently occurs in remote resource-limited settings and has been designated a priority for research development by WHO. CCHF viral load at baseline is an important prognostic indicator that is associated with more severe disease and prolonged viraemia, whilst urine is rarely RT-PCR positive. CCHF is characterised by a pro-inflammatory and early adaptive host immune response. ROTEM analysis has demonstrated that the coagulopathy in CCHF is predominantly in clot development/stabilisation suggesting platelet dysfunction.

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Declaration

Apart from the assistance as outlined in the acknowledgments above, the work described is my own and has not been submitted for a degree or other qualification to this or any other University.

Contents:

Publications and presentations

Crimean-Congo haemorrhagic fever related articles published during the Fellowship:

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Oral Presentations:

1. CCHF Therapeutics (invited speaker and session chair) – WHO CCHF R&D Stakeholder meeting. Annecy Feb 2018.
2. CCHF interaction of clinical course, viral dynamics and host immune response. Royal Society of Medicine - Colt Prize winner. London 2017.
3. CCHF clinical management (invited speaker and session chair). 2nd International CCHF conference. Thessaloniki. 2017.
4. Fighting Ebola on the frontline. British Society Gastroenterology Annual conference. Liverpool May 2016.
5. Ebola Virus Disease – Clinical Lessons Leaned (invited WHO speaker). RKI/WHO EDCARN expert meeting. Berlin April 2016.
6. Ebola Virus Disease – Early days, challenges and setting the standard. Haywood Club Conference. Dec 2015.
7. Clinical features of EVD – What we know now? British Infection Society Ebola Conference. Manchester Nov 2015.
8. Building resilience for Ebola. Defence Security Equipment International Exhibition. London Sept 2015.

Podcasts/Public engagement:

1. Innovation in viral haemorrhagic fevers. Military medicine podcast. https://podtail.com/sv/podcast/the-military-medicine-podcast/
2. BMJ Talk learning. Ebola podcast: Medical guidance and lessons from west Africa. https://soundcloud.com/bmjpodcasts/ebola-medical-guidance-and-lessons-from-west-africa-with-dr-tom-fletcher?in=bmjpodcasts/sets/bmj-best-practice-clinical
3. SciFri – BBC Radio Merseyside. https://www.lstmed.ac.uk/news-events/events/lstms-dr-tom-fletcher-on-scifri-radio-merseyside.

List of abbreviations used in this thesis

A10	Amplitude 10 minutes
ALT	Alanine transaminase
APTT	Activated partial thromboplastin time
AST	Aspartate transaminase
BUN	Blood urea nitrogen
CCHF	Crimean-Congo haemorrhagic fever
CCHFV	Crimean-Congo haemorrhagic fever virus
CDC	Centers for disease control and prevention
CFT	Clot formation time
CHF	Crimean haemorrhagic fever
CL-3	Containment level 3
CL-4	Containment level 4
CRP	C-reactive protein
CCL	Chemokine (C-C) ligand
CK	Creatine kinase
CI	Confidence interval
CRF	Case record form
Ct	Cycle threshold
CT	Clotting time
CXCL	Chemokine (C-X-C) ligand
DAMPs	Damage-associated molecular patterns
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
ECDC	European centre for disease prevention and control
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ET-1	Endothelin 1
EVD	Ebola virus disease
FDA	Food and drug administration
FFP	Fresh frozen plasma
GPC	Glycoprotein
HFRS	Haemorrhagic fever with renal syndrome
HPS	Hantavirus pulmonary syndrome
HIV	Human immunodeficiency virus
HCT	Haematocrit
IATA	International air transport association
IEC	International electrotechnical commission
IL	Interleukin
IFN	Interferon
IQR	Interquartile range
INR	International normalised ratio
IP	Interferon gamma-induced protein
ISARIC	International severe acute respiratory and emerging infection consortium
ISO	International organisation for standardisation
qSOFA	Quick sequential organ failure assessment
LDH	Lactate dehydrogenase
LV	Lassa virus

MCP	Monocyte chemoattractant protein
MCF	Maximum clot firmness
MIP	Macrophage inflammatory protein
MIF	Macrophage migration inhibitory factor
MoDREC	Ministry of Defence research and ethics committee
NCR	Non-coding region
NEWS	National early warning score
OMU	Ondokus Mayis University
OMUH	Ondokus Mayis University Hospital
OROV	Oropouche virus
OUT	Ovarian Tumour
PAMPs	Pathogen-associated molecular patterns
PBS-T	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
PEP	Post-exposure prophylaxis
PHE	Public health England
Plts	Platelets
PPE	Personal protective equipment
PT	Prothrombin time
RANTES	Regulation on activation, normal T cell expressed and secreted
REC	Research and ethics committee
ROTEM	Rotational thromboelastometry
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
STFS	Severe fever with thrombocytopenia syndrome virus
SD	Standard deviation
sVCAM	Soluble vascular cell adhesion molecule
sICAM	Soluble intercellular adhesion molecule
SSI	Severity scoring index
SGS	Severity grading system
SPSS	Statistical package for the social sciences
SPSD	Solution for preliminary serum dilution
TBE	Tick-borne encephalitis
TLR	Toll-like receptor
TNF	Tumour-necrosis factor
TMB	Tetramethylbenzidine
ULNV	Upper limit normal value
ULN	Upper limit normal
USSR	Union of Soviet Socialist Republics
UVRI	Ugandan virus research institute
VHF	Viral haemorrhagic fever
VL	Viral load
WBC	White blood cell
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 Research questions and aims.

Research Questions:

Crimean-Congo haemorrhagic fever (CCHF) is an emerging infectious disease threat and causes significant mortality:

1. What is the relationship between CCHF viral dynamics, immune response and disease severity?
2. Does severe/fatal CCHF disease correlate with a sepsis model of cytokine network deregulation?

Specific Aims:

A cohort of adults with PCR positive CCHF in Turkey was recruited to:

1. Determine the viral load dynamics in serum and urine over the course of clinical infection (day 1-7, 14 and 30).
2. Investigate the hypothesis that: (1) initial viral load and (2) rate of viral load reduction are correlated with disease severity and clinical outcome.
3. Investigate the host immune response to clinical infection through serial measurement of cytokine/chemokine levels and quantitative serological response, related to disease severity and clinical outcomes.
4. Test the hypothesis that severe/fatal CCHF is associated with a deregulated cytokine network characterised by an exaggerated pro-inflammatory cytokine response (IL-1βeta, IL-6, IL-8 and tumour necrosis factor alpha [TNF-α], and reduced IL-12).
5. Undertake ROTEM analysis alongside conventional coagulation testing to investigate the coagulopathy in CCHF.

General Aims: This extensive preliminary study also aimed to establish a platform and framework for future therapeutic intervention studies in CCHF, including identification of surrogate markers and development of a library of clinical isolates for future research.

1.2 Background

1.2.1 Crimean-Congo Haemorrhagic Fever Virus (CCHFV)

CCHFV is an arbovirus of the *Orthonairovirus* genus in the *Nairoviridae* family. The family *Bunyaviridae* was reclassified by the ICTV in 2017 to the order *Bunyavirales.* The 5 previous genera: *Hantavirus; Nairovirus; Orthobunyavirus; Phlebovirus;* and *Tospovirus* [1] are now novel viral families. *Bunyavirales* are predominantly vector borne viruses transmitted by specific mosquitoes, ticks, midges, or biting flies. No arthropod vector has been demonstrated for hantaviruses that are transmitted to humans predominantly via inhalation of virus-contaminated excreta from specific rodent reservoir hosts [2]. *Tospoviruses* only infect plants but several viruses within the remaining families cause significant severe disease in humans such as Rift Valley fever.

CCHFV is an enveloped RNA virus and virions are spherical and approximately 90-100nm in diameter [3]. They have a lipid bilayer envelope approximately 5–7nm thick, through which protrude glycoprotein spikes 8–10nm in length. Negative stain electron microscopy has shown CCHF virions to be distinct from other viruses within the *Bunyavirales* order, as they possess very small morphologic surface units, with no central holes unlike other *Bunyavirales* [3] (Figure 1.1).

CCHFV has a negative sense single stranded RNA genome, and in common with all *Bunyavirales*, is divided into 3 distinct segments designated the small (S), medium (M) and large (L). The total genome size is approximately 20kb in length (larger than other *Bunyavirales*) and the S segment codes for the nucleocapsid protein, the M segment codes for the 2 envelope glycoproteins (Gn and Gc), and the L segment codes for the RNA dependent polymerase [4]. The small segment also codes for a non-structural S protein (NSS) in the positive sense[5] (Figure 1.2).

The first complete viral genomes were determined in 2006, and a high degree of genetic diversity amongst CCHFV strains is observed with strains clustering in 7 distinct groups, based on S segment sequences [4]. The 7 groups or clades have a strong geographical distribution with clades Africa 1, Africa 2 and Africa 3 containing strains predominantly from Africa. Europe 1 has strains from Turkey, Russia, Kosovo, Greece, Bulgaria and Albania, whilst Europe 2 has a single AL92 strain isolated in Greece. Asia 1 has strains isolated from the Middle East, whilst Asia 2 has isolates from China, Tajikistan, Kazakhstan, and Uzbekistan.

Bunyavirales families	
Nairoviridae	CCHFV Dugbe virus
Phenuiviridae (previously Phlebovirus)	Sandfly fever Sicilian virus, Toscana virus Rift Valley Fever virus Severe fever with thrombocytopenia syndrome virus (SFTSV) Bhanje virus
Peribunyaviridae (previously Orthobunyavirus)	Bunyamwera virus California encephalitis virus - La Crosse virus Oropouche virus (OROV) Bwamba Fever virus (BWAV)
Hantaviridae	Hemorrhagic fever with renal syndrome (HFRS) Hantavirus Pulmonary Syndrome (HPS)

Table 1.1 Major Bunyavirales causing disease in Humans

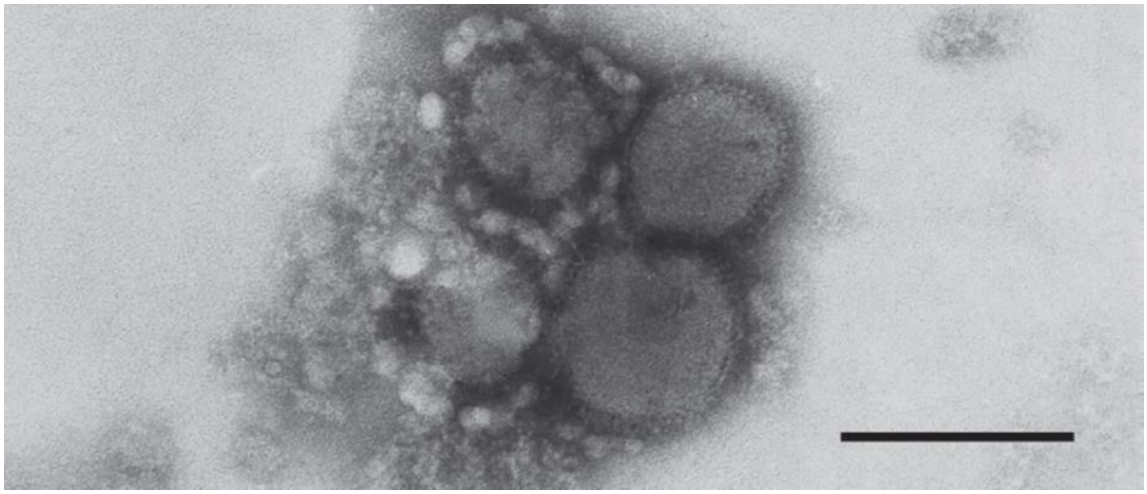


Figure 1.1 Electron micrograph of negatively stained particles of Crimean-Congo haemorrhagic fever virus (CCHFV). The bar represents 100 nm. (From C. S. Schmaljohn. Bunyaviradae, Fields virology 2014)[1]

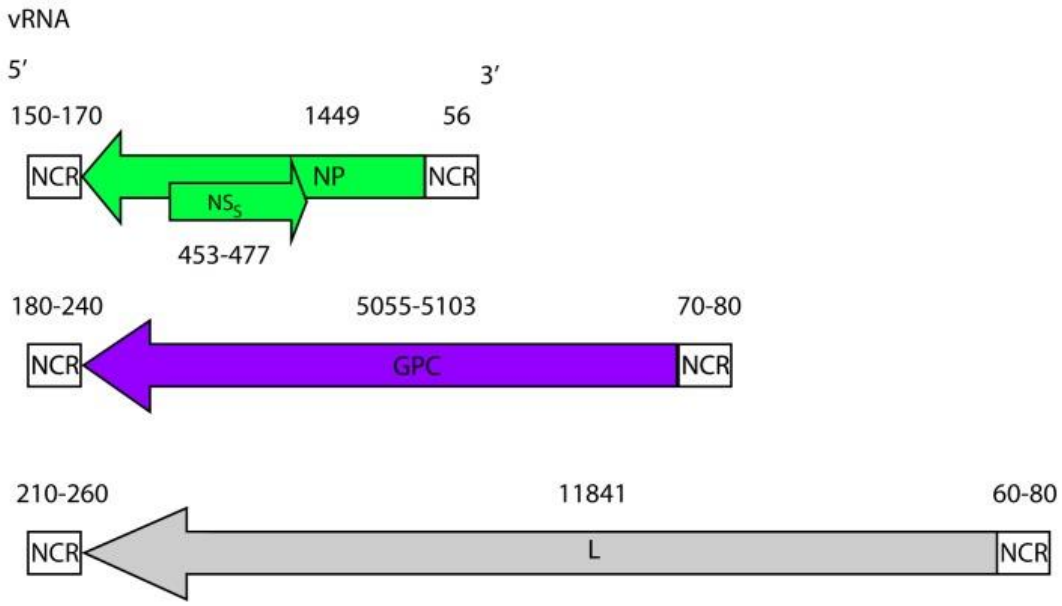


Figure 1.2 CCHFV genome (From Zivcec et al. Molecular Insights into Crimean-Congo Hemorrhagic Fever Virus. Viruses 2016) [6]

1.2.2 Emergence of CCHF

Crimea

CCHF was first recognised and described in the Spring and Summer of 1944, when a febrile illness was observed in up to 200 Soviet army troops and agricultural workers in Crimea. Small clusters of similar cases were then reported annually in the same region. An investigation and description of the first outbreak was made by military physicians and subsequently by a scientific expedition led by Professor Mikhail Chumakov of the USSR Academy of Medical Science [7]. Chumakov went on to be a pivotal figure in developing the oral polio vaccine. He had also previously been part of a team that discovered the viral aetiology of tick-borne encephalitis (TBE), accidentally becoming infected after cutting his finger during an autopsy, leading to a permanent loss of hearing and paralysis of the right arm [8].

Chumakov and colleagues named the syndrome Crimean haemorrhagic fever (CHF) and reported that it consisted of fever and a range of haemorrhagic complications, mainly gastrointestinal, combined with hypotension and shock. Initial case fatality rates were 8% in 1944 and it is believed that CCHF emerged in the Crimea at this point due to favourable environmental factors during the wartime period including: neglected agricultural lands; introduction of a new non-immune population and a local resurgence of the hare population due to reduced hunting. Russian investigators also recognised the similarity between CHF and earlier 12th century reports of a severe febrile illness that was reported by the Tadzhik physician and philosopher Dzhurdzhani [9]. In Uzbekistan a similar haemorrhagic disease was known as 'karakhalak' meaning black death [10].

Initial research by Russian scientists to develop an animal model and establish the aetiology of CHF were unsuccessful despite attempts in white mice, guinea pigs, rabbits, monkeys and cats. In 1945-46 psychiatric patients were directly injected with samples from clinical cases in the Crimea, as pyrogenic therapy. Samples from a pulverised and filtered suspension from Hyalomma marginatum nymphs were also injected resulting in a characteristic CHF illness [11]. The first reliable animal model in the USSR was developed in 1967 in the Institute of Poliomyelitis and Viral Encephalitides in Moscow utilizing newborn white mice brains, allowing serial passage and virus isolation. Russian investigators also reported that the majority of cases were occurring in milk maids and agricultural workers, with many reporting that they frequently removed and crushed engorged ticks from their cattle between their fingers. They also reported the role of ground feeding birds as tick hosts, recognising the risk of the potential introduction and establishment into new geographical areas [12].

Congo

In 1956 in the Democratic Republic of Congo (Belgian Congo), Dr Ghislaine Courtois was the head of the provincial medical laboratory in Stanleyville, when he reviewed a 13 year old boy with fever, headache, nausea, vomiting, back pain, arthralgia and photophobia [13]. He took a blood sample, injected this into 3-day old mice, and then began serially passaging the virus through new-born mouse brains. A month later he did the same with his own sample when he became unwell with a febrile illness. He passed the sample through a seitz filter to establish it as a virus, but as he had no dry ice or freezer available he was forced to maintain the virus through serial passage (35 and 46 times) [13].

He subsequently sent the mouse brains to the East African Virus research institute in Entebbe Uganda where they remained in a freezer for 4 years. These were then found by John Woodall in 1961 and after he was unable to confirm the identity of the virus using haemagglutination-inhibition and complement fixation, the samples were sent to the Rockefeller Foundation Virus Laboratory in New York. Dr Jordi Casals tested it there against reference strains, finding it identical to another unnamed strain from Uganda and contacted Dr Courtois who asked for it to be called Congo virus. Details of the virus were first published in 1967 in the East African Medical Journal [14], including 12 cases of CCHF, of which 5 were due to laboratory exposure at the Uganda Virus Research Institute (UVRI). The International Committee for the Taxonomy of Viruses named it Crimean-Congo Haemorrhagic Fever virus (CCHFV), although a number of reports still refer to it as Congo-Crimean Haemorrhagic fever.

Turkey

In 2002 health facilities in Tokat located in the central Anatalolia region of Turkey reported that a significant number of patients were being seen with fever, rash and thrombocytopenia. In 2003 a joint investigation was undertaken by the Ministry of Health and the Ministry of Food, Agriculture and Livestock, which found that the majority of patients reported a history of tick bite and worked in animal husbandry [15]. A common clinical syndrome was recognised including non-specific febrile illness, thrombocytopenia, leucopenia and elevated liver enzymes, that initial reports suggested was treated successfully with tetracyclines. In 2002, serum samples from 19 patients were sent to the WHO Collaborating centre for Rickettsial Reference and Research Laboratory in Marseille where 7 of them were reported as acute Q fever, while 8 of them were reported as previous Q fever cases [16].

Cases continued in 2003 with increased haemorrhage and a lack of tetracycline response also reported. Samples were then sent to the WHO Collaborating Centre for Arboviruses and Viral Haemorrhagic Fevers in France, which diagnosed CCHF, that was confirmed by testing of previous legacy samples [17]. RT-PCR positive cases were also confirmed by Karti et al [18] at the

same time at Karadeniz technical University in collaboration with the Centers for Disease Control and Prevention, Atlanta.

1.2.3 Epidemiology

Although CCHFV has been isolated from a range of tick species, only ticks from the from the genera *Dermacentor, Hyalomma*, and *Rhipicephalus* have been confirmed as vectors. Its spread is aligned with the distribution of its main vector *Hyalomma* ticks with *Hyalomma marginatum* considered to be the most important vector for transmission of CCHF virus in Europe [19]. Ticks also act a natural reservoir for CCHFV, tending to occur in areas of warmer climate with high fragmentation of the landscape vegetation. Although they can exist in a range of habitats, in Europe the Alps and Balkan mountains seem to act as natural barrier to their distribution [20].

Climatic factors such as mild winters, reduced rainfall and changes of reservoir species can result in increase in suitable habitats for ticks [21]. Human behaviour can also have a direct influence on emergence through the neglect of agricultural land or deforestation, and changes in hunting activities that may result in increased wildlife and ticks; this was suggested to have happened prior to its emergence in Russia and also in Turkey [15,21]. Both larvae and nymphs feed without leaving the host, and as a result may be attached to hosts for long periods. This long attachment allows them to be passively transported by migratory birds: this has also been suggested to play a role in in the possible spread of CCHFV, as initially suspected by Russian researchers and later demonstrated in Turkey [22,23]. Introduction of *Hyalmoma marginatum* ticks to the United Kingdom by migratory birds has also been demonstrated [19].

Antibody production and transient viraemia lasting 7-15 days has been demonstrated in a range of wild and domestic vertebrates that have an asymptomatic infection. Cattle, sheep and goats have been investigated in the largest seroprevalence studies, with combined international data reporting CCHFV seroprevalences of 19%, 24% and 28% respectively. Antibody production to CCHFV has not been detected in birds or reptiles with the exception of ostriches and guinea fowl [24–28]. CCHFV has a wide geographical distribution with cases mainly occurring in Asia, the Middle East, South-Eastern Europe and Africa. Since its emergence in 2002, Turkey has been the epicentre of activity worldwide reporting up to 1000 cases annually. Russia, Iran, Pakistan and Afghanistan are the other countries where the disease has been reported most often in the last decade [15]. More than 30 countries worldwide have reported human cases with Spain recently reporting cases of autochthonous transmission [29].

Africa	Asia	Middle East	Europe
Democratic Republic of Congo [14]	Pakistan [30]	Iran [31]	Russian Federation [32]
South Africa [33]	Afghanistan[34]	Iraq [35]	Georgia [3637]
Nigeria [38]	Tajikistan [39]	United Arab Emirates[36]	Bulgaria [40]
Senegal [41]	Uzbekistan[42]	Saudi Arabia [43]	Macedonia [44]
Uganda [14]	Kazakhstan[45]	Oman [46]	Albania [47]
Tanzania [33]	China [48]		Kosovo [49]
Mauritania [50]	India [51]		Turkey [15]
Kenya [52]			Greece [53]
Burkina Faso [54]			Spain [29]
Namibia [55]			Hungary [56]
Sudan [57,58]			Serbia [59]
Central African Republic [60]			
Ghana [61]			
Benin [62]			
Madagascar [63]			
Mali [64]			

Table 1.2 . Countries that have reported cases of CCHF or have serological evidence in Humans. NB in addition Niger [65], Azerbaijan [66], Armenia [67], Hungary [56], Somalia [68], Zimbabwe [33] and Egypt [69] have serological evidence of CCHF in animals.

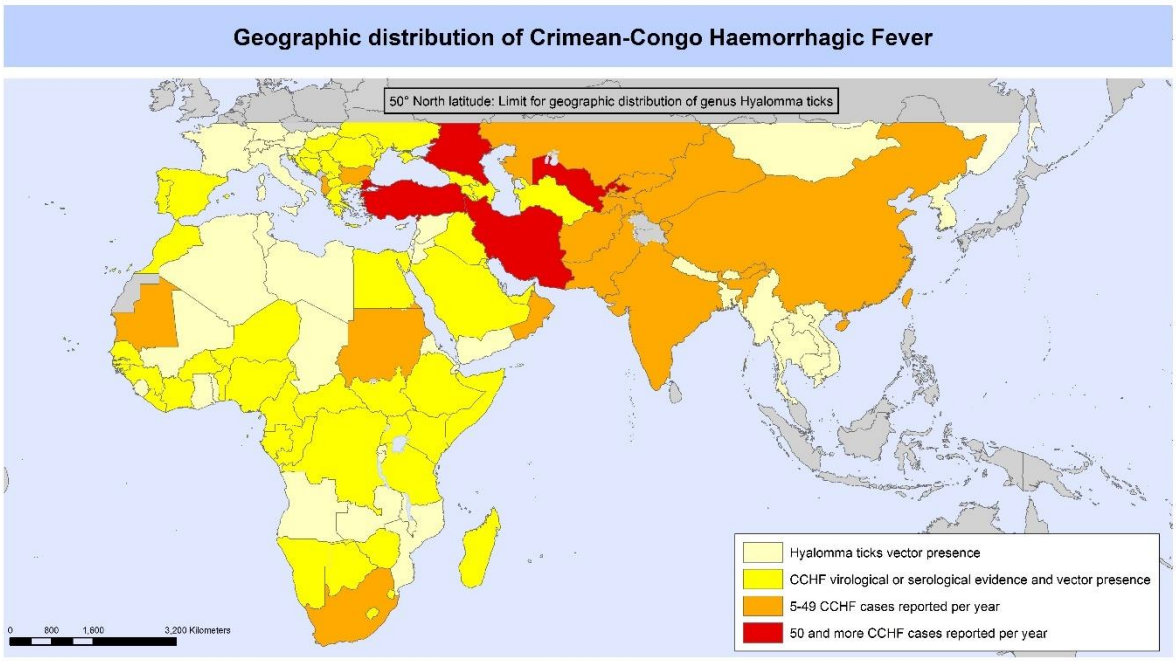


Figure 1.3. Worldwide distribution of CCHF (modified from WHO with permission)

Genotype	Countries
Africa 1	Western Africa (Senegal, Mauritania)
Africa 2	Central Africa (Uganda, Democratic Republic of Congo)
Africa 3	South Africa, Nigeria, Uganda
Asia 1	The Middle East (Iran, Iraq, Oman, United Arab Emirates), Pakistan, Afghanistan
Asia 2	Far East and Central Asia (Tajikistan, Uzbekistan, Kazakhstan, China), Iran
Europe 1	Eastern Europe (Albania, Kosovo, Bulgaria, Russia, Turkey, Greece), Iran
Europe 2	Greece (AP92 strain), Turkey

Table 1.3 Geographic distribution of CCHF virus according to S-segment analysis. The phylogenetic grouping of S RNA segments illustrates that the pattern of genetic diversity observed is largely related to the geographical distribution of the viruses. (Leblebicioglu H uptodate.com)

1.3 Pathogenesis of Crimean-Congo Haemorrhagic Fever

Although CCHF is widely distributed and increasingly reported, the pathogenesis of disease is poorly understood. This is mainly due to the necessity to perform research in advanced biosafety laboratories (termed containment level 4 (CL-4) in the UK), the poor availability of animal models and the occurrence of infections in areas where research facilities are limited. The role of ribavirin antiviral therapy is controversial, and improved understanding of disease pathogenesis should inform new approaches to improving clinical outcomes.

When CCHFV penetrates into the body it will be encountered first by the innate immune system that will act 'quasi-automatically' generating inflammation [70,71]. The key cells involved are monocytes/macrophages, neutrophils, eosinophils, basophils and natural killer cells. These leucocytes are activated by binding of molecules from CCHFV, pathogen-associated molecular patterns (PAMPs) or from necrotic cells, damage-associated molecular patterns (DAMPs) to pattern recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs)s, nbod-like receptors, RIG-like receptors and C-type lectin receptors (Figure 1.4). TLRs are the best studied and trigger a cascade of activation generating release of inflammatory cytokines.

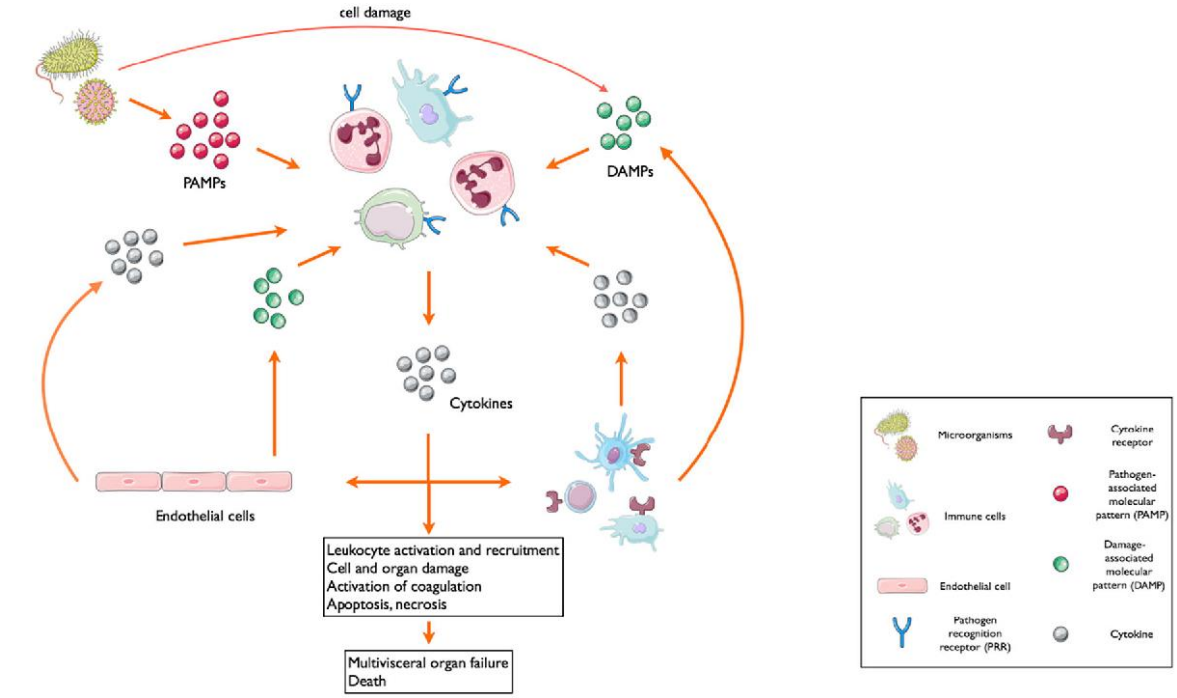


Figure 1.4 – Cytokine cascade during sepsis. (From Chousterholm et al. Seminars in Immunopathology 2017) [70].

CCHFV amplifies in dendritic cells and macrophages that facilitates spread and the replication process for CCHFV is similar to those of many other enveloped viruses [72]. The viral glycoproteins are believed to be responsible for recognition of receptor sites on susceptible cells. Viruses which attach to receptors on susceptible cells are then internalized by endocytosis, with replication occurring in the cytoplasm [73]. Virions mature by budding through endoplasmic reticulum into cytoplasmic vesicles in the Golgi region, which are presumed to fuse with the plasma membrane to release virus [74,75].

One of the first and most effective immune defences against viral infections are Type 1 Interferons. After cells detect viral replication products through complex signalling pathways, Type 1 IFN is synthesized and secreted, that further upregulates interferon-stimulated genes (ISGs) [76,77]. The action of the ISGs activates a complex host defence program that results in an antiviral state in the cell and neighbouring cell [78]. Interferons also act to induce apoptosis and cellular resistance to infection, inhibit cell proliferation and activate natural killer cells and the adaptive immune response [79,80]. *Bunyavirales*, including CCHFV, have developed mechanisms to evade the IFN induced antiviral state. CCHFV has been shown to delay the production of interferon in infected cells by impeding activation of the Interferon regulator factor 3 pathway. The L segment of CCHFV contains an ovarian tumor (OTU) domain, which is thought to suppress immune signalling, modulate virulence and counteract the effects of IFN induced antiviral proteins [81]. This domain has also been shown to inhibit NF-Kb [81], a major rapidly acting transcription factor that regulates genes responsible for both the innate and adaptive immune response, including apoptotic genes [82].

The regulation of apoptosis through suppression or induction during acute viral infection is also important for virus survival and dissemination [83]. Apoptosis can be started by an extrinsic (death receptor mediated) or intrinsic pathway (mitochondria mediated). CCHFV has been shown to induce caspase-3 and modulate both intrinsic and extrinsic pathways in cell cultures [5,84]. Elevated apoptotic markers have been demonstrated in a paediatric cohort of CCHF [85], and modulation of apoptotic gene expression facilitating apoptosis, shown in a cohort of 20 patients in Turkey [86]. In this study dominant extrinsic pathway activation, mostly with TNF family members was observed, with additional intrinsic pathway activation in severe and fatal cases.

In animal models CCHFV initially replicates in blood, liver and spleen, before wider dissemination to lung, kidney and brain [87,88]. CCHF is characterized by haemorrhage and increased vascular permeability indicating the involvement of endothelial cells, and CCHFV has been demonstrated to have direct and indirect effects on endothelial cells upon infection [89]. Indirect effects are through virus mediated host immune response (e.g. cytokines) or directly by

virus infection and replication in endothelial cells [90]. Endothelial cells also play a key role in haemostasis influencing coagulation, platelet adhesion and activation, fibrinolysis, platelet disintegration and vasoregulation [91].

1.3.1 Pathogenesis – CCHF Viral load

Viral load at admission has been shown to be an important prognostic indicator in Ebola virus disease (EVD) [92,93] and Lassa Fever [94]. A correlation between initial serum viral load and disease severity/outcome in CCHF has also been shown in 11 studies to date that have reported CCHF viral load data (Table 1.4). Cevik et al [95] and Duh et al [96] first reported viral load data from Turkey and Kosovo respectively in 2007 that showed CCHF viral loads were higher in fatal than surviving cases. Both studies demonstrated that a VL greater than 1 x 10^9 copies/ml was indicative of a fatal outcome. This has been confirmed by a number of further studies [97–99], some of which also demonstrated differences in viral load by severity score. A limited number of studies have investigated viral loads serially with daily sampling. The largest by Bodur et al [100], measured viral loads daily in 50 patients, 10 of whom received ribavirin. By day 3 of admission 50% of patients had cleared virus, with only 1/42 having a positive PCR on day 6. This is consistent with Cevik et al's [95] first report and Ergunay et al's [101] more recent data demonstrating that virtually all patients had cleared viraemia within 6 days of admission, with patients being admitted 3-5 days after onset of symptoms.

The largest study undertaken evaluating CCHF VL at baseline was published by Hasanoglu et al [99] in 2016. It included 126 patients (14 with fatal disease) recruited over 6 years from Ankara. They demonstrated significant differences in mean viral load between fatal and surviving cases (4.6×10^9 copies/ml vs 8.3×10^7 copies/ml, $p<0.005$), and also that blood product transfusion was used significantly more frequently in patients who had viral load >10^8 copies/ml, as was haemodialysis. One report by Papa et al [102] also demonstrated that CCHF viral loads were higher in primary than secondary cases, although the sample size was small and all primary cases had severe disease with haemorrhage. The viral load dynamics in urine remain unclear, with potential infection prevention control and diagnostic implications. Prolonged viraemia in both serum and urine (>30 days post disease onset) has been demonstrated in one report from Kosovo, that included patients with serial positive/negative/positive results [103]. CCHFV has also been detected in one other study in urine and the saliva of plasma CCHFV PCR positive patients [104].

Publication	Study period	Study design	Country	Number of participants	Day of tests	Results
Cevik et al. 2007 [95]	2006	Prospective	Turkey	36 patients (9 fatal)	Daily	In 8 /9 patients with fatal outcomes, VL >1x 10 ^{9} copies/ml In 25/26 patients with non-fatal outcomes, VL < 1x10^{9} copies/mL (P <0.001). All surviving patients cleared viraemia by day 6 hospital admission
Duh et al. 2007[96]	2001, 2003, 2005	Retrospective	Kosovo	24 patients (9 fatal)	Admission	VL fatal group log10 9.25 (1.8 × 10^{9}) VL survivor groups log10 6.91 (8.1 × 10^{6})
Wolfel et al. 2007 [105]	NS	Retrospective	South Africa, Iran, Pakistan	21 patients	Mix	Correlation between viral load and duration of symptoms
Papa et al. 2007 [102]	2003-2006	Retrospective	Albania	12 patients	Admission	VL higher in primary than secondary cases
Saksida et al. 2010[97]	2001-2007	Retrospective	Kosovo	46 patients (11 fatal)	Admission	VL fatal group: 9.31 log10 copies/ml VL survivor group: 6.31 log10 copies/ml (p<0.001). Fatal groups 9/10 VL >1x10^{8} copies/ml Survivor groups 30/32 VL <1 x 10^{8}copies/ml (p<0.001). Higher VL in severe (mean, 6.97 log10 copies/ml) than moderate disease course (mean, 5.65 log10 copies/ml) (p= 0.006)
Bodur et al. 2011 [100]	2006-2008	Prospective case control study	Turkey	50 patients, 10 ribavirin (2/10 fatal) 40 control (6/40 fatal)	Daily	Positive VL by day admission and ribavirin Day 1: 10/10 and 40/40 Day 2: 9/10 and 30/39 Day 3: 5/9 and 22/39 Day 4: 2/9 and 12/37 Day 5: 1/9 and 4/34 Day 6: 1/8 and 0/34
Kubar A et al. 2011 [106]	NS	Prospective	Turkey	26 patients – given hyperimmune serum	Daily	Low VL (<10^{7}copies/ml): 8/11 cleared by 38h, 11/11 cleared by 72h High VL (10^{8}copies/ml): 15/15 pos 48 hrs, 6/15 pos 96h, 1 pos 144h.

Kaya S et al 2014[98]	NS,	Prospective	Turkey	31 patients (11 fatal)	Daily 7 days	VL in fatal group 5.5 x 10^9 copies/mL VL survivor group 5.7 x 10^8 copies/mL ($p<0.001$) CCHF VL was significantly and positively correlated with serum IL-6, TNF-α and blood CRP levels, PT, aPTT, INR values and DIC scores In contrast to fatal patients, a clear declining trend was observed for serum VL in survivors
Ergunay K et al 2014 [101]	2012	Prospective	Turkey	20 patients	Daily	17/20 RNA pos Viraemia lasted median 3 days admission
Papa et al. 2015 [107]	2011-2013	Retrospective	Bulgaria and Turkey	35 patients (4 fatal) 16 controls	Admission	VL mean 1.47x10^7copies/ml VL correlated with day of illness
Hasanoglu et al. 2016 [99]	2008-2013	Prospective	Turkey	126 (14 fatal)	Admission	VL survived 8.3 × 10^7 copy/ml VL fatal group 4.6 × 10^9 copy/ml No patient survived VL >2 × 10^9 copy/ml Blood product transfusions were significantly used more in patients who had viral load >10^8 copy/ml. Patients with 10^8 copy/ml or higher viral load: Diarrhoea, headache, unconsciousness, bleeding, seizures, and haemodialysis were significantly more frequent ($p<0.05$).

Table 1.4. Published studies investigating CCHF viral load

1.3.2 Pathogenesis – CCHFV antibody

Antibody production against CCHFV is hypothesized to be important in survival, with weak or no antibody response demonstrated in fatal cases. Early studies using indirect immunofluorescence assays demonstrated the presence of IgG and IgM antibodies on days 7 to 9 of illness with the highest antibody titre reached in the second to third week of illness [26,108]. In a study of 20 Turkish CCHF cases, nucleoprotein-specific IgM antibodies were detectable a median of 2-3 days after disease onset, followed by GPC-specific IgM (4-6 days) and IgG antibodies (5-6 days) [101].

Duh et al [96] evaluated CCHF IgG and IgM levels in 43 samples from 24 patients showing that quantitative IgG correlated with viral loads, with none of the 9 fatal cases (day of illness, median 6, range 2-9) developing positive IgG titres. In contrast 9/21 acute samples from survivors with moderate and severe disease in the same cohort were positive for IgG but taken at later time points (median 9, range 2-13). Only one of the 9 survivor samples with a positive IgG result was taken less than 9 days after disease onset (at day 6). No correlation with IgM positivity and death or viral load was demonstrated.

Saksida et al [97] also investigated CCHF antibody responses in admission samples from 46 patients from Kosovo. No IgG response was detected in 11 fatal cases (median sample time 6 days, range 3-9 days), whilst 5/34 survivor samples had a positive IgG titre (median 6 days, range -2-12 days, positive IgG results occurred at days 6,6,9,12 and 12 of illness). There was no relationship between IgM status and outcome in this study. Kaya et al [98] evaluated serial antibody responses in 31 patients with CCHF in Turkey, including 11 fatal cases. They showed that all survivors demonstrated a positive IgG titre within 9 days of onset, whereas none of the fatal cases did by the same timepoint.

The role of cell-mediated adaptive immunity in CCHF infection has not been well studied. Goedhals et al [109] recently demonstrated CCHF memory T cell responses persisting for up to 13 years post infection in 11 survivors. Increased circulating CD3+ and CD8+ T cells has been shown in fatal CCHF disease[110], with little known about functional T cell responses (Th1/Th2) and their role in disease severity and outcome.

1.3.3 Pathogenesis – Cytokines

Cytokines are a broad category of small proteins involved in cell signalling, that have a broad range of functions including immunomodulatory. They can be divided into several categories: interleukins; chemokines; interferons; tumor necrosis factor; and growth factors [70,111]. A tightly regulated cytokine network is crucial for elimination of invading pathogens and restricting excessive tissue damage [112,113], and sepsis syndrome in general has been shown to develop when this initial host response becomes amplified and deregulated [114–117]. Release of inflammatory cytokines induces new cytokine production and release, and this 'cytokine storm ' or 'cytokine cascade' is likely responsible for the many diverse and local effects of the 'sepsis syndrome' [70]. This has been demonstrated in a range of viral and bacterial infections, and was recently popularised largely in the context of avian influenza [118].

Numerous pro-inflammatory cytokines have been identified during sepsis with IL-1β, IL-6, IL-12, and IL-17 being of crucial importance. However despite large progress in the research of sepsis utilising the 'omics' (genomic, proteomic, metabolomic and transcriptomic), therapeutic clinical trials targeting cytokines and the immune response in sepsis have failed [70]. The failure of immunomodulatory trials and current lack of clear understanding in the organisation of host response reflects the fact that septic patients do not represent a homogeneous group. Patients have different pathogens, presentations and levels of severity that make direct comparisons and clinical trials focussed on immune response modification challenging.

Cytokines in viral haemorrhagic fevers/CCHF

In viral haemorrhagic fevers, a common pathogenic feature is the ability of the virus to disable the host immune response by attacking and manipulating macrophages and dendritic cells (90,91). In severe cases of CCHF, it has been suggested that this, combined with the deregulation and excessive pro-inflammatory cytokine release leads to toxic endothelium effects, increased vascular permeability, multi-organ failure, and shock [87]. A significant number of studies have evaluated the role of cytokines in CCHF, most frequently through analysis of baseline admission samples and direct comparison of severity groups or fatal/non-fatal disease (Table 1.5). Fourteen of the 18 studies identified are from Turkey, with the largest study including 80 patients. Only one study of 31 patients evaluated a panel of 4 cytokines daily for 7 days, demonstrating IL-6 and TNF-α were consistently higher in fatal cases. A recent study of 52 patients evaluated a panel of cytokines every 3 days (119 samples in total) demonstrating that IL-6, IL-8, IL-10, IL-10/ 12, IFN-γ, MCP-1, and MIP-1β were higher in patients with CCHF than controls, and that IL-6 and IL-8 were higher in the first 5 days of illness in fatal cases.

Evaluation of the combined data from a number of studies broadly demonstrates that high serum levels of pro-inflammatory cytokines, particularly IL-6 and TNF-α, have been implicated

as negative prognostic factors in CCHF/ VHF [97,98,119–124]. IL-10 is an anti-inflammatory cytokine that has also been shown to be higher is severe/fatal disease in some studies [119,123,125], but not in others [120,126]. IL-12 production has demonstrated a mixed picture in relationship to CCHF disease and severity compared to controls [97,121,126]. Other studies have investigated endothelial adhesion mediators showing sVCAM-1 and sICAM-1 to be higher in severe cases [127,128], and other markers of endothelial dysfunction to be higher in haemorrhagic cases [129].

Overall the ability to draw prognostic inferences from these studies is limited in most cases due to their retrospective design, incomplete data sampling, size and the evaluation of cytokines at only one time point. Patients with CCHF present at different stages of illness and whilst baseline evaluation of cytokines may be to useful to incorporate variables into prognostic severity scores, larger longitudinal studies are required to provide greater insights into the dynamic host immune response over time. Sample integrity for cytokine measurement is also important, and benefits from early processing and storage in ultra-low freezers. Repeated freeze-thaw cycles of legacy samples taken in different outbreaks may affect cytokine results and the ability to draw clear and valid conclusions. Due to the number of cytokines involved, use of multiplex would be extremely beneficial, but technically difficult due to the highly pathogenic nature of VHFs.

Publication	Study period	Study design	Country	Number of participants	Day of tests	Results
Papa et al. 2006 [130]	2003	Retrospective	Albania	16 patients (1 fatal) 26 controls	Diagnostic samples – day 5	TNF-α and IL 6 in cases raised vs control IL10 raised in fatal case
Ergonul et al. 2006 [120]	2002-2004	Retrospective	Turkey	30 patients	Admission	IL6 and TNF-α higher in fatal cases – no difference in IL-10 IL6 and TNF-α correlated with higher DIC scores, IL10 neg correlated
Pinar et al. 2008 [131]	2006	Not reported	Turkey	51 patients 30 controls	Admission	Neopterin higher in cases than controls (15 x) and higher in fatal cases (3x)
Bodur et al. 2010 [127]	2007	Prospective case -control	Turkey	75 patients 88 controls	Admission	sVCAM-1, sL-selectin and MIF higher CCHF vs controls sICAM-1, sP-selectin, sE-selectin, and VEGF lower CCHF vs controls sVCAM-1 and sICAM-1 higher in severe cases, VEGF lower in severe cases
Ozturk et al. 2010 [128]	2007-2008	Not reported	Turkey	61 patients (8 fatal)	Admission and discharge	HA, sICAM-1, sVCAM-1, and VEGF-A levels higher in fatal cases. HA, sICAM-1 higher in acute vs conv samples.
Saksida et al. 2010 [97]	2001-2007	Retrospective	Kosovo	46 patients (11 fatal)	Admission	IL10 IFN gamma and TNF-α higher fatal outcome, and linear relationship with VL IL12 reduced in all patients
Ozsurekci et al. 2013 [126]	2010-2011	Not reported	Turkey	34 children 36 adults 30 controls	Admission	IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL- 6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, and IL-22 – no difference in adults or children vs controls or difference in severity in children. Higher IL-2, IL-5, IL-9, IL-12 p70, and IL-13 in fatal adults (n=3) vs survivor adults
Erturk et al 2014. [132]	Not reported	Not reported	Turkey	42 patients 40 controls	Not reported	Resistin higher in CCHF vs controls, and recovery time.
Kaya et al. 2014 [98]	Not reported	Prospective	Turkey	31 patients (11 fatal)	Longitudinal – daily for 7 days	IL-6 and TNF-α higher in fatal cases
Sancakdar et al. 2014[121]	Not reported	Not reported	Turkey	52 children 34 controls	Acute samples <7 days onset disease	IL-10 and ET-1 levels higher in severe group vs non-severe IL-6 and TNF-α levels higher in sever than controls IL-12 no diff controls or severity
Arasli et al. 2015. [133]	2010-2011	Retrospective Cases vs controls	Turkey	29 adults 32 children	Not reported	Adults - median CCL2, CCL3, CCL4, CXCL8, and CXCL9 higher CCHF vs controls Children median CCL4 and G-CSF levels higher CCHF vs controls
Papa A et al 2015 [107]	2011-2013	Retrospective	Bulgaria and Turkey	35 patients (4 fatal) 16 controls	Admission	CXCL10 (IP-10) raised in cases and correlated with VL.

Parlak et al. 2015. [122]	2012-2013	Prospective case control	Turkey	80 patients (5 fatal) 100 controls	Admission	IL-2, IL-6, IL-10 higher in patients vs controls. IL-10, IL-6 higher severe and fatal cases
Yilmaz et al. 2015[134]	2008-2011	Retrospective	Turkey	53 patients 36 controls	Admission	Resistin and ghrelin levels higher in severe vs non-severe CCHF
Demirpence et al. 2016 [135]	Not reported	Prospective case control	Turkey	59 patients 28 controls	Admission	Presepsin higher in CCHF vs controls and higher in severe CCHF disease
Papa et al. 2016. [123]	2003-2011	Retrospective	Turkey, Albania and Greece	29 patients 16 controls	Admission	IP-10 and MCP-1 raised in severe vs non-severe cases IL-1β, IL-6, IL-10, IP-10, MCP-1, TNF-α raised in fatal cases Low levels of RANTES in fatal cases
Arslan et al. 2017.[129]	2010-2012	Prospective	Turkey	73 patients	Admission and 5th day admission	Endothelial dysfunction markers higher in CCHF vs controls * ADMA, ET-1, TM, vWF, ICAM-1). TM and vWF >in haemorrhagic cases.
Ergonul et al 2017 [124]	2007-2009	Retrospective.	Turkey	52 patients (119 samples) 10 controls	Admission and every 3 days	IL-6, IL-8, IL-10, IL-12, IFN-γ, MCP-1, and MIP-1β higher in CCHF than controls. IL-6 and IL-8 higher in first 5 days in fatal cases.

Table 1.5. Published studies investigating cytokines/chemokines in CCHF

1.4 Clinical presentation of Crimean-Congo Haemorrhagic Fever

The clinical spectrum of illness and disease severity in patients with CCHF is broad, and it is estimated that up to 88% of infections may be sub-clinical [136]. In highly endemic areas such as Tokat and Sivas provinces in Turkey, the CCHF IgG seroprevalence has been shown to be as high as 12.8% in rural populations [137]. Case fatality rates in laboratory confirmed cases have been around 5% in Turkey, independent of ribavirin use, but consistently higher in other countries and have exceeded 50% in smaller outbreaks [15,138,139]. This is in part due to differences in access to supportive care, but probably also reflects different severity of disease in different cohorts/settings due to limited surveillance activities and access to diagnostic support. Turkey has an active community engagement program with annual education campaigns to communities and healthcare facilities in endemic areas, combined with access to a network of reference laboratories. As a result, it identifies a significant number of mild severity CCHF cases annually, often in patients that present with a history of only tick bite and fever, and mild thrombocytopenia. By contrast in Pakistan a significant proportion of cases have travelled from Afghanistan for treatment, and reflect a more severely unwell cohort, often with haemorrhage and higher associated case fatality rates [140].

The majority of the patients with CCHF report a history of tick bite (70%)[141]. The incubation period ranges from 1-13 days (typically 1-3 days after tick bite), and has been shown to be shorter in fatal cases [50,142]. Data from a large cohort in Turkey (n=1670) showed that the most common complaints at presentation were fever (90%), fatigue (90%), headache (70%), myalgia (70%) and nausea (65%). Haemorrhagic manifestations were reported in 23% of patients at admission[141]. Leucopenia (88.9%), thrombocytopenia (93.2%) and elevated transaminases (85.9%), LDH (75.8%) and CK (65.9) were the most common laboratory abnormalities at presentation.

The first study evaluating prognostic indicators in CCHF was by Swanepoel et al [143] in South Africa in 1989. They reported that the occurrence of any of the following factors during the first 5 days of illness was >90% predictive of a fatal outcome: Leucocyte counts >10 x 10^9/L; platelet counts <20 x 10^9(/L; AST >200 IU/L; ALT >150IU/L; APTT >60 seconds; and fibrinogen <110 mg/dL. Subsequent studies have evaluated a range of clinical and laboratory valuables prognostically [99,144–147]. The main clinical features associated with mortality by multivariate analysis include impaired consciousness [144,148,149], diarrhoea [149,150] and haemorrhagic manifestations [148,150]. Elevated APTT[147–150] and elevated ALT [147,150,151] are the most consistently abnormal laboratory parameters in multivariate analysis, with raised LDH an independent predictor of death in 2 studies [147,150] and platelet count <20 x 10^9/L a predictor in one study [148].

Two severity scoring systems for CCHF have been developed, utilising these and additional laboratory parameters (Table 1.6). The severity grading score (SGS) system was first reported by Bakir et al in 2012[152], and then validated by data from over 400 patients from multiple centres in Turkey [153]. It divides patients into low (0%), intermediate (20%) or high-risk group (100%) for mortality based on 12 parameters in the first 5 days of illness (ALT, AST, LDH, WBC, platelets, PT, INR, aPTT, age, bleeding, hepatomegaly and organ failure). The second CCHF severity scoring system developed is the severity scoring index (SSI) reported by Dokuzoguz et al [154] that grades patients as mild, moderate or severe disease at admission utilising 5 parameters (platelets, aPTT, fibrinogen, bleeding and somnolence). The SSI system has the advantage in that it is based on admission data, and therefore can be used in patients that present after 5 days of illness, but also has the downside of including fibrinogen which is not widely available or routinely measured in all patients. A simpler scoring system for CCHF, incorporating CCHFV viral load, that has consistently been shown to be associated with severe and fatal disease [96,97,99], may be useful.

Swanepoel criteria - 1st 5 days of illness - Any of following >90% predictive fatal outcome	**Severity grading system** - 1st 5 days of illness - 0-4 Low risk - 5-8 Intermediate risk - >9 High risk	**Severity scoring index** - At admission - 0-2 Mild - 3-9 Moderate - 10-13 Severe
Platelet count ≤20 X 10^9 /L	Platelet count ≥100,000 cells/μL = 0 ≥50,000, <100,000 cells/μL = 1 <50,000 cells/μL = 2	Platelet count (×10^3/mm^3) >150 = 0 150–50 = 1 49–20 = 2 <20 = 3
APTT ≥ 60 seconds	aPTT (seconds) <70 = 0 ≥70 = 1	aPTT (seconds) ≤34 = 0 35–45 = 1 46–59 = 2 >60 = 3
Aspartate transaminase ≥200 U/L	Aspartate transaminase < 5×ULNV = 0 ≥ 5×ULNV = 1 Lactate dehydrogenase <3×ULNV = 0 ≥3×ULNV= 1	Fibrinogen level, mg/dL ≥180 = 0 179–160 = 1 159–120 = 2 <120 = 3
Alanine transaminase ≥150 U/L	Alanine transaminase <ULNV = 0 ≥ULNV = 1	Bleeding No = 0 Petechia = 1 Ecchymosis = 2 Bleeding = 3
White blood cells ≥10,000 cells/μL	White blood cells <10,000 cells /μL = 0 ≥10,000 cells /μL = 1	Somnolence No = 0 Yes = 1
Fibrinogen <110 mg/dL.	Organ failure No = 0 Yes = 1	
	Bleeding No = 0 Yes = 1	
	Age <60 = 0 >60 = 1	
	Prolongation of PT <3 s = 0 ≥3s, <6 s = 1 ≥6s = 2	
	Hepatomegaly No = 0 Yes = 1	
	INR <1.6 = 0 ≥1.6 = 1	

Table 1.6 CCHF severity scoring systems

1.5 Treatment of Crimean-Congo Haemorrhagic Fever

The case management of CCHF is largely supportive with a focus on the provision of blood component therapy to prevent bleeding and critical care support when it is required for severe cases. Haematological support is predominantly with platelet and fresh frozen plasma transfusions, guided by the clinical picture and laboratory results. Additional measures used to reduce the risk of haemorrhage include avoidance of non-steroidal anti-inflammatories and anti-platelet medication, and stress-ulcer prophylaxis provided to severe cases or those with a history of peptic ulcer disease [155]. The majority of patients receive supplemental intravenous fluid and electrolyte replacement, but the volumes required and the electrolyte imbalance that occurs in most cases is significantly less than in other VHFs such as EVD [92,156].

Higher levels of supportive care are required as the disease progresses with severe cases requiring access to intensive care support including renal replacement therapy and mechanical ventilation. Somnolence and confusion are noted to be poor prognostic indicators with cerebral oedema and haemorrhage reported in fatal cases [72,157]. Ventilatory support can be provided safely and results in better outcomes, but presents a risk of aerosol generation and must be carefully planned and managed to prevent nosocomial transmission [157,158]. Renal replacement therapy is also regularly provided to patients with CCHF in Turkey [159,160] with standard precautions applied in dialysis units. Surgical interventions to control haemorrhage such as endoscopy have been undertaken successfully in endemic settings (Sener Barut personal communication 2016), as have a number of caesarean sections in pregnant woman, although this has also led to nosocomial infections [161]. Antibiotics are not routinely administered, and it has been shown that co-infection with bacteraemia only rarely occurs and is predominantly related to intravenous catheter related infections [162].

Ribavirin is a synthetic nucleoside analogue that shows in vitro activity against some RNA and DNA viruses [163]. Its mechanism of action against CCHFV is unknown but it has demonstrated in vitro activity [164] and had mixed results in animal models [165,166]. Although it has not been approved for use in CCHF by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA), it is listed as being of potential benefit in CCHF by the World Health Organisation. It is included on the WHO model list of essential medicines, due to its clearer evidence base in the treatment of Lassa fever and Argentinian Haemorrhagic fever and the early reports of benefit in CCHF [167–169]. Since the emergence of CCHF in Turkey and Iran there have been a number of studies evaluating ribavirin treatment, although most of these have been retrospective and consequently affected by confounding factors and bias [146,170,171]. One randomised controlled trial of 136 patient undertaken between 2004-2007 failed to show any reduction in mortality, or positive effect on clinical or laboratory parameters [172]. A subsequent

meta-analysis by Ascioglu et al [173] included 8 studies and 731 patients and did not demonstrate that ribavirin conferred any survival benefit or additional benefit such as shorter hospital stay, earlier improvement of laboratory values or decreased requirement for blood products. A recent meta-analysis undertaken by the Cochrane infectious diseases group concluded that the benefit of ribavirin in treating CCHF was unknown and highlighted the risk of bias from non-randomised observational studies [174].

Ribavirin does have a more established role in post-exposure prophylaxis (PEP) for healthcare workers that have a had a high risk CCHFV exposure such as a needle stick injury. A large multicentre retrospective cross-sectional study undertaken in Turkey, covering the years 2012-2014 identified 51 HCW exposures [175]. Post-exposure ribavirin prophylaxis was administered after 19/44 cases of known CCHF exposure, with 0/19 developing laboratory confirmed infection or clinical disease, compared to 18/25 untreated cases that developed laboratory-confirmed infection, of which 11/18 developed clinical disease. Underlying bias may have influenced cases selected for ribavirin PEP, and it is not possible to adjust for other factors that may have influenced the results, but it may be that PEP is effective when there is a smaller inoculum post-exposure, but it is ineffective in treating the high virus loads seen in clinical disease.

Hyperimmune serum has been evaluated in one study of 26 patients [106]. Although the trial had no control group it did report a low case fatality rate in 15 high risk patients (with a CCHFV viral load $>10^8$ copies/ml) and requires further evaluation in a randomised clinical trial. Corticosteroids have also been utilised in severe cases with a suggested rationale of reducing an exaggerated host immune response and 'cytokine storm'. Retrospective analysis of 281 cases of which 44 received ribavirin and corticosteroids suggested a possible benefit of corticosteroids in severe disease, but is limited by potential confounding factors and missing data [154]. Steroids have also not been shown to clearly reduce mortality in sepsis and septic shock generally in larger meta-analyses [176–178].

Chapter 2 Methods

2.1 Scientific and ethical approval

Research and Ethical committee (REC) approval for the study was obtained in advance by the Ministry of Defence Research and Ethical Committee (MoDREC) in the UK (571) and Ondokus Mayis University REC in Samsun, Turkey (OMU KAEK 739).

2.2 Study design - Prospective observational cohort study.

The study was conducted between May 2015 and August 2016 in Turkey at Ondokus Mayis University (OMU) Hospital, Tokat State Hospital and Tokat University Hospital. Both university hospitals are Ministry of Health designated specialist CCHF referral centres in close proximity to the hyperendemic region in mid-Anatolia. CCHF cases in Turkey are seasonal occurring predominantly during May-September allowing for focused and efficient screening procedures.

Figure 2.1 CCHF hyperendemic region in Turkey by state with study sites.

Ondokus Mayis University is a large 1000-bed teaching hospital in Samsun, Northern Turkey on the Black Sea coast. It serves a city of over half a million people in Samsun and is a major port city. It is famous historically as the location where Mustafa Kemal Ataturk started the Turkish war of Independence on the 19th May 1919. The Infectious diseases department at Ondokus Mayis University Hospital is large and receives referrals of patients with severe/complicated CCHF from Amasya, Tokat and Corum. Tokat State and University Hospitals are large tertiary hospitals located in the CCHF hyperendemic region and collectively manage the most confirmed CCHF cases annually of any centre worldwide.

Suspected cases are admitted directly from the Emergency Department to the units and also through a regional referral network. During the study adults with suspected CCHF were identified and managed according to Turkish national guidelines at the Infectious Diseases units at each hospital. Infectious diseases specialists at each hospital were responsible for the clinical

management of patients. At each location a study team was trained by the Principal investigator, and study sites opened sequentially to allow training and quality assurance in recruitment and sample processing (Ondokus Mayis University Hospital – 15th May 2015, Tokat State Hospital – 22nd June 2015 and Tokat University Hospital – 29th June 2015).

2.3 Participant Recruitment

Study materials, patient information leaflets and consent documents were available in both English and Turkish. Participants or an appropriate relative/guardian were approached by the research team staff trained in consent procedures, were provided with a participant information leaflet, and provided written informed consent. Participants were generally recruited on the first or second day of hospital admission, with first sampling occurring on day 1-3.

Inclusion criteria for participants:

1. Suspected or confirmed infection with CCHF admitted to hospital

Exclusion criteria for participants:

1. Refusal by participant, parent or appropriate representative.
2. Age <18 years

Children (aged <18yrs) were not recruited as participants in this study. Paediatric cases of CCHF do occur but are less frequent and managed by paediatricians, as opposed to adult CCHF infectious diseases specialists. Although recognising the importance of studying the pathogenesis of CCHF in all age groups, recruitment of paediatric participants presents additional challenges and has a different clinical disease pattern. It would require specific sub-group analysis and would need a larger multi-centre study for adequate sample size and power that was not within the scope of this fellowship**.**

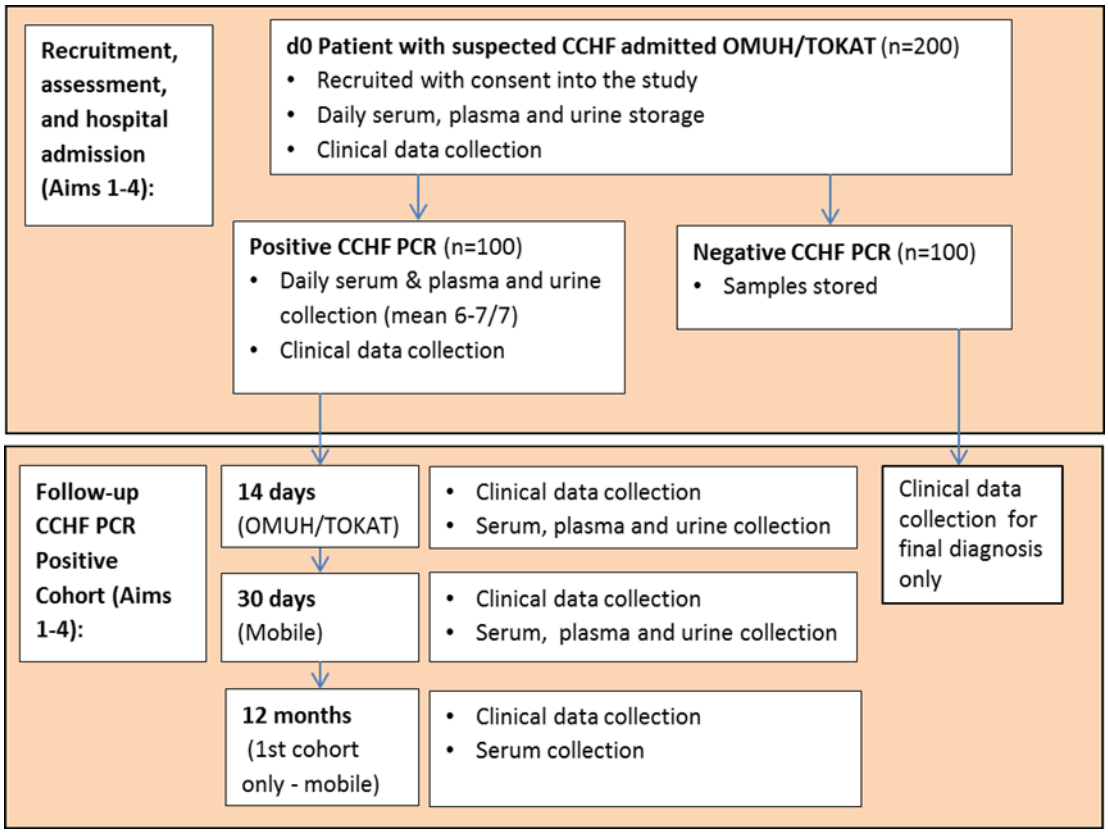


Figure 2.2 Recruitment, investigation and follow-up of CCHF cohort

Follow-up

Participants were invited to additional follow-up at 14, 30 and 365 days with repeat sampling at each time point. Follow up was either in the hospital infectious diseases department or mobile in the community dependent on the geographical location of the patient.

2.4 Participant sampling

The volume of blood collected was dependent on the weight of the participant, to prevent excessive volume sampling from small adults (Table 2.1). Blood samples required for medical management had priority over samples taken for research tests, and attending physicians decided on the combination of routine blood tests that were undertaken daily on clinical grounds, disease severity and hospital location. Whenever practical, research samples were timed to coincide with routine clinical sampling. Daily venepuncture is part of the standard protocol for the medical care of this group and dependent on the study site was undertaken by the PI, infectious diseases fellows or nurses responsible for in-patient management. Research samples were taken at the same time (during the same blood draw), after routine clinical samples. Post-phlebotomy site bleeding was managed as per standard protocols.

Weight	Samples at recruitment (R)	Serial samples (S) daily	Convalescent samples 14, 30 days	Total volumes of blood taken
>40kg	4mls EDTA blood 5mls blood in serum(clotted) tube 4mls Citrated tube 3mls blood in blood RNA tube 10ml Urine	4mls EDTA blood 5mls blood in serum(clotted) tube 4ml citrated tube 10 mls Urine	4mls EDTA blood 5mls blood in serum(clotted) tube 10 mls urine	Maximum any day: 16mls (0.38mls/kg) Maximum any 4 weeks: 96mls (2.4mls/kg)

Table 2.1 Study sampling schedule

2.5 Laboratory development and sample processing

Research laboratories were established in the Infectious Diseases departments of Ondokus Mayis University Hospital and Tokat State Hospital. Ultra-low freezers (-80^{0}C), temperature-controlled bucket centrifuges (Thermo Scientific Sorvall ST 16R, ClikSeal lids) and Class I and Class II Biological Safety Cabinets were sourced for each laboratory. Laboratory protocols were developed in conjunction with RIPL PHE and training undertaken by the PI of one ID specialist at each site. Samples were processed in Turkey, stored at -80^{0}C and then shipped to the CL-4 laboratory at PHE Porton on dry ice by World Courier as Category A samples. Permission from the Health and Safety Executive in the UK was obtained prior to importation.

Figure 2.3 Research Laboratory OMU

Figure 2.4 Research Laboratory Tokat State Hospital

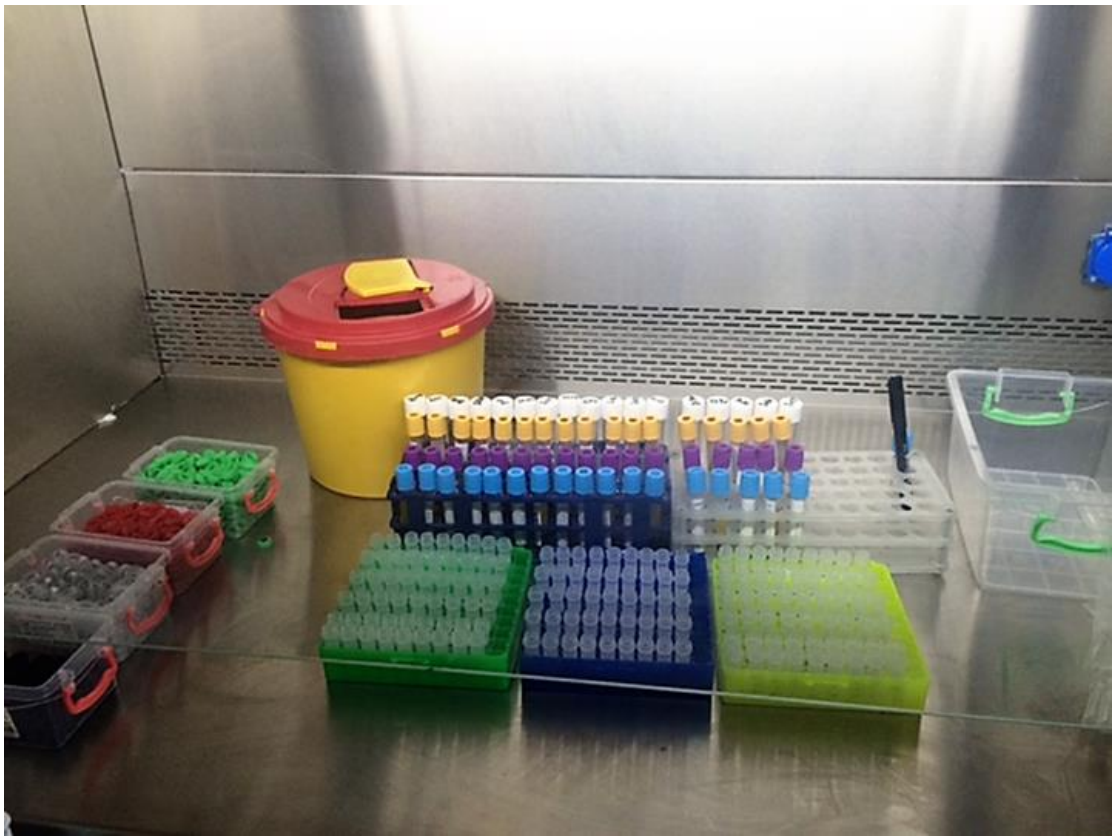


Figure 2.5 One study day's samples in Class I Biological Safety Cabinet (17 participants)

Figure 2.6 Samples in ClikSeal Buckets in Sorvall 16R centrifuge.

Figure 2.7 Mobile follow-up of participants at 1 year after acute infection. The top-left picture shows a participant's house in a remote rural village in Tokat where communities continue to live above their livestock.

2.6 Project safety and Infection prevention and control precautions

Safety of the research project was given the highest priority in full recognition that CCHFV is a Hazard Group/Category 4 Pathogen. Study team and participant safety was given the highest priority from the point of sample collection at the bedside through to laboratory processing and analysis at OMUH/Tokat and then at PHE Porton.

Well-established hospital protocols were used to collect specimens and strict adherence to collection protocols, biosafety and adequate personal protective equipment (PPE) were maintained. Biosafety procedures were as per local policy/guidance (WHO standard for Viral Haemorrhagic Fever) and were applied to the collection, transport, storage and laboratory handling of research samples. It was ensured that there was adequate equipment to complete the task safely and adherence to universal precautions and WHO best practices for phlebotomy at all times [179]. The study team underwent training and mentorship by the Study PI to ensure safety was maintained at all time. Any new safety issues identified were brought to the attention of the Study PI.

All samples collected were labelled as per standard procedure with appropriate identification and hazard labelling. All samples sent to external laboratories were anonymized with unique coded identifiers to protect the identity of the patient at the point of enrolment. International guidance (IATA) was adhered to for the transport of samples to PHE Porton (World Courier Ltd). The Health and Safety Executive in the UK provided advance approval for importation of samples to PHE Porton.

2.7 Data management and analysis plan

Prospective data on patient demographics, co-morbid conditions, medications, clinical features, clinical laboratory test results and treatment were collected on a standardised proforma developed by the PI. The case record form (CRF) is an adaptation of the ISARIC/WHO viral haemorrhagic fever CRF and was piloted in July 2014 at OMU. Data was then entered into the ISARIC electronic database, with subsequent quality assurance data checking against original CRFs subsequently.

All participants had CCHF severity assessment undertaken by Swanepoel criteria, severity grading score (SGS) (9) and severity scoring index (SSI) scoring systems (Table 1.6). Participants presenting more than 5 days after onset of illness had an SSI score undertaken at admission. They were categorized as ‘mild’ if they had none of the Swanepoel parameters, were graded as mild by SSI and low risk by SGS. The ‘moderate/severe group’ included all patients graded as moderate/severe (SSI) or intermediate/high risk (SGS) or with Swanepoel criteria. Missing

laboratory variables scored as zero in scoring systems. Participants diagnosis was through PCR routinely performed within 24 hours at the Public Health Laboratory in Samsun.

Sample size calculations were based on proposed differences in viral load and pro-inflammatory cytokines (Il-12) between patients in mild/moderate and severe/fatal groups. Published viral load and cytokine data were used. Based on prior observational research at OMU Hospital, a recruitment rate of >90% was anticipated with a total study sample size of 100 patients.

Based on the severity scoring system and data published by Saksida et al [97], and assuming a 60:40 split between the 'mild/moderate' and 'severe/fatal` groups:

1. Mean (SD) log10 (viral load) levels in the "mild/moderate" and "severe/fatal" groups were estimated to be 5.654 (1.603) and 7.903 (1.510) respectively; the proposed sample size would detect a difference of this magnitude or greater with >95% power.
2. Mean (SD) log10 (IL-12) levels in the "mild/moderate" and "severe/fatal" groups would be 1.541 (0.220) and 1.301 (0.505) respectively; the proposed sample size would detect a difference of this magnitude or greater with 79.1% power.
3. Sample size would have 90% power to detect a correlation of 0.316 ($R^2 \approx 10\%$) or greater between log10(viral load) levels and log10 (IL-12) levels.
4. All of these calculations assumed conventional two-sided significance levels of 5% and utilised Gpower version 3.1.

Descriptive analyses are reported as frequencies (proportions) for categorical variables and means (standard deviation or 95% confidence interval (CI)) or medians (ranges) as appropriate for continuous variables. Categorical variables were compared between sub-groups with the Fisher exact test. Continuous variables measured at admission were compared using Student t-tests or the Mann-Whitney U-test/Kruskal-Wallis test as appropriate. Bonferroni correction was applied to counteract risk of Type 1 errors through multiple comparison. Hypothesis testing of cytokines were grouped by severity/low platelets with subsequent analysis at $p<0.025$. Continuous variables measured longitudinally were compared using linear regression models with robust standard errors and adjustment for clustering of measures within patients when required. Correlation between variables was measured by Pearson correlation coefficient. No imputation for missing data was made due to small sample sizes. Hypothesis tests were two-tailed ($p<0{\cdot}05$) and analyses were performed using the SPSS (version 24), Stata (version 14) and GraphPad Prism (version 7.03) computer packages.

Fellowship time frame	Pre-	First year				Second year				Third year				Post-
Site visit and submission ethics committees (PI and OMU Supervisor)														
Study preparation														
Main study period (Aims 1-4) 1st cohort recruitment, investigation and follow-up. (PI and project team at OMU/TOKAT)														
Second study period (Aims 1-4) Recruitment, investigation and follow-up (inc 12 month follow up 1st cohort) (PI and project team at OMU)														
Laboratory analysis (Aims 1-4) Quantitative Viral load analysis Serological profiling Luminex flow cytometry (PI and team at PHE Porton)														
Data Analysis Preparation for presentation, publication, PhD														

Figure 2.8 Fellowship timetable

2.8 Role of the Chief Investigator/Wellcome Trust Fellow

I was fully responsible for study conception/design and submission for ethical approval in the UK and Turkey. I trained and mentoring all study teams in Turkey and established two new CCHF research laboratories with guidance and SOPs developed in conjunction with PHE Porton. I supervised recruitment and consent of participants at study sites and undertook >50% of participants daily blood sampling. I processed and separated >90% of urine and blood samples in Turkey and was responsible for storage and sample transfer to PHE Porton. I undertook all ROTEM laboratory analysis in Turkey and worked with the laboratory team in PHE Porton on PCR, ELISA and luminex assays for 10-15% of samples. I supervised CRF data entry at all study sites and inputted all data to the study's electronic database, undertaking all quality assurance checks. I undertook all data analysis and interpretation with support from supervisors, and guidance from medical statisticians at LSTM and UoL.

Chapter 3: Demographics and descriptive clinical data

3.1 Study participants

One hundred and forty-four participants with suspected CCHF were recruited into the study at the three study sites. One hundred and four were found to be CCHFV PCR positive at the Public Health Reference laboratory in Samsun and continued in the study. Two participants subsequently withdrew consent for continued sampling and follow-up.

Sixty-six of 104 (63.5%) participants included in the study were male and the mean age was 50.0 (SD 15.9) years. A history of tick bite was reported by 73/104 (70.2%) and 87/104 (83.7%) were admitted directly to the study site, with 17/104 (16.3%) being transferred from another healthcare facility. The median time from onset of symptoms until hospital admission was 4 days (range 1-11 days), and in those with a history of tick bite, the median time from bite until onset of symptoms was 3 days (range 1-13 days). The median length of hospital admission was 8 days (range 2-16), with 3/104 fatal outcomes (CFR 2.9%) (Table 3.1).

The majority of participants (91/100) resided in rural CCHF endemic areas, with 93/99 having visited the countryside in the 2 weeks prior to onset of symptoms (including 9 participants that did not reside in rural areas). The majority of participants reported a history of close animal contact (81/99), and 47/97 reported direct contact with animal blood or tissue. 3/97 participants reported a history contact with sick individuals in the 2 weeks prior to onset of symptoms, but none of these individuals were admitted with confirmed CCHF. Most patients had no significant past medical history: Diabetes mellitus (11/100); cardiovascular disease (10/100); and pulmonary (5/100) disease were the most commonly reported chronic conditions (Table 3.2).

	N (%) (n=104)
Gender (%):	
- Male	66 (63.5)
- Female	38 (36.5)
Age (years)	
- Mean (SD)	50 (15.9)
Tick bite (%):	
- Yes	73 (70.2)
- No	30 (28.8)
- Unknown	1 (1.0)
Mode of admission (%):	
- Direct	87 (83.7)
- Hospital transfer	17 (16.3)
Time from symptom onset to admission (days)	
- Median (range)	4 (1-11)
Time from tick bite onset symptoms (days)	
- Median (range)	3 (1-13)
Length of admission (days)	
- Median (range)	8 (2-16)
Risk factors (%):	
- Live rural area	91/100 (91)
- Visit the countryside last 2 weeks	93/99 (93.9)
- Contact animal blood/tissue	47/97 (48.5)
- Close contact animal	81/99 (81.8)
- Contact with sick individuals last 2/52	3/97 (3.1)
CCHF severity score (%):	
- Mild/low	63 (60.6)
- Moderate/severe	41 (39.4)
Highest qSOFA score during admission	
<2	61 (59.2)
>2	43 (40.8)
Fatal outcome (%):	
- Yes	3 (2.9)
- No	101 (97.1)

Table 3.1 Demographic characteristics of CCHFV PCR positive participants

Past medical history	Total n (%)
Cardiovascular disease	10/100 (10.0)
Pulmonary disease	5/100 (5.0)
Renal disease	3/100 (3.0)
Liver disease	1/100 (1.0)
Cerebrovascular disease	0/100 (0.0)
Diabetes	11/100 (11.0)
Obesity	2/100 (2.0)
Rheumatology	3/100 (3.0)
Dementia	1/97 (1.0)
HIV /Lymphoma/cancer	0/100 (0.0)

Table 3.2 Past medical history of CCHFV positive participants

3.2 Clinical and Laboratory features of CCHF participants

At admission the combined CCHF severity scoring systems demonstrated that 63/104 (60.6%) were classified as mild, and 41/104 (39.4%) were classified moderate/severe severity. Of the 41 participants classified as moderate/severe, 19/41 were graded as moderate/high risk by all 3 scoring systems, 8/41 by 2 scoring systems and 14 by one scoring system (Table 3.3, Figure 3.1).This overlap and variation in scoring systems is demonstrated by the Figure 3.1, showing the severity allocations of the 41 participants in the moderate/severe group be each scoring system.

There was no significant difference in age between severity groups (mild 51.8 years (SD 16.6) vs moderate/severe 47.2 years (SD 14.3, p= 0.14). The lowest platelet count recorded by patients during admission is another pragmatic disease severity indicator (a count of $<50 \times 10^9$/L level), utilised in Turkey as a threshold for transfer to tertiary CCHF centres. Fifty-six participants (53.8%) had a platelet count of $<50 \times 10^9$/L during admission with 22/104 (21.2%) having platelets measured at $<20 \times 10^9$/L. In the moderate/severe group 35/41 participants had a platelet count of $<50 \times 10^9$/L, with 22/63 in the mild group having a platelet count of $<50 \times 10^9$/L ($p<0.001$).

CCHF Severity Score	Total n=104
Severity grading score (SGS):	
- Low risk SGS ≤ 4	83 (79.8)
- Intermediate risk SGS 5-8	19 (18.3)
- High risk SGS ≥ 9	2 (1.9)
Severity scoring index (SSI):	
- Mild 0-2	65 (62.5)
- Moderate 3-9	39 (37.5)
- Severe 10-13	0 (0.0)
Swanepoel criteria:	
- Non-severe	77 (74.0)
- Severe/poor prognosis	27 (26.0)
Combined CCHF severity score:	
- Mild or low risk	63 (60.6)
- Moderate/severe or intermediate/high risk	41 (39.4)
Lowest platelet count:	
- <50 x 10^9/L	56 (53.8)
- <20 x 10^9/L	22 (21.0)

Table 3.3 CCHF severity scores of CCHF positive participants

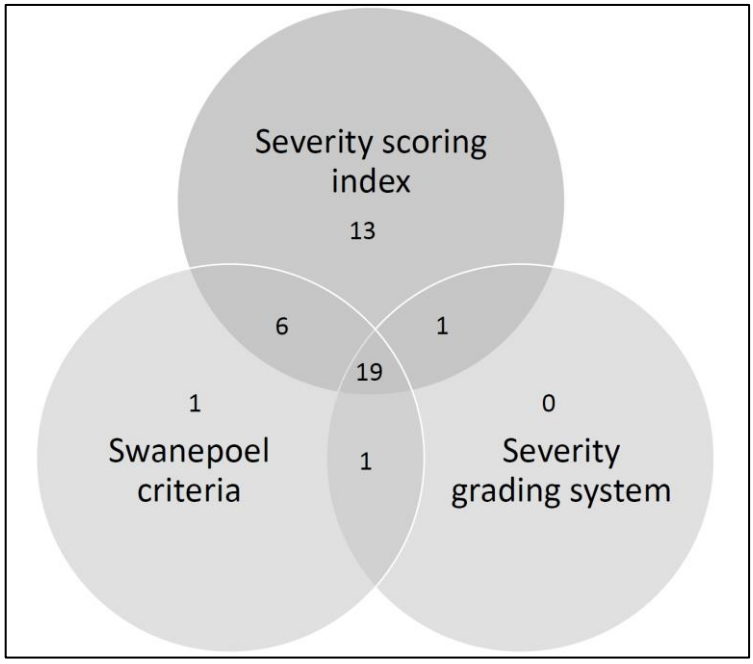


Figure 3.1 Venn diagram of severity scores for 41 participants in moderate/severe group

3.2.1 Clinical and laboratory features at admission

At admission to hospital the most common complaints were of fever (88.2%), headache (75.7%), fatigue (74.8%), nausea (74.5%) and myalgia (72.3%). Anorexia was also common (63.3%), with vomiting reported by 52.4% and diarrhoea by 33% of participants. Bleeding at the time of admission was reported by 14/103 (13.6%) participants (Figure 3.2,Table 3.4). On comparison of clinical features at admission between severity groups, anorexia was more common in severe/moderate severity group (29/37 vs 33/61, p=0.02) as was petechiae (6/40 vs 0/62, p=0.003), bruising (4/40 vs 0/61, p=0.02), splenomegaly (4/28 vs 0/49, p=0.02) and bleeding (14/40 vs 0/63, p<0.001).

The admission mean temperature was 37.5^{O}C (SD 0.83). Only 38/104 (36.5%) of participants had a temperature recorded of ≥38 ^{0}C at admission, with 50/104 (48.1%) a temperature recorded of ≥37.5^{0}C. Mean pulse, systolic and diastolic blood pressure were within normal limits, with mean respiratory rate elevated at 21.2 (SD 2.4). Diastolic and systolic blood pressure was lower in severe/moderate disease (p=0.07, p=0.04) , with temperature lower in severe/moderate disease compared to mild disease (37.2 (0.8) vs 37.6 (SD 0.8), p=0.02). Although NEWS score was higher (median 2, range 0-8 vs median 1, range 0-5) in the moderate/severe group, this was not statistically significant (p=0.66).

Admission blood results are demonstrated in Table 3.5. Blood urea nitrogen (BUN) levels were elevated (median 22 mg d/L) on admission, but creatinine was within normal limits (median 0.81 mg/dL). Glucose levels were also elevated (median 110 mg/dL) as was alanine transaminase (median 44 U/L) and aspartate transaminase levels (median 79 U/L). Creatine kinase(CK) levels and lactate dehydrogenase (LDH) levels were elevated (CK median 298 U/L, LDH (n=37) median 553 U/L), whilst amylase and bilirubin were within normal limits.

Haematology laboratory results demonstrated that thrombocytopenia (platelet count <150 x 10^{9}/L) was not universal at admission with 20/103 participants having platelets counts >150 x 10^{9}/L. Median platelet count was reduced at 104 x 10^{9}/L with a range of 6 – 231 x 10^{9}/L. White blood cell count was also reduced (median 2.7 x 10^{9}/L), as was haemoglobin (13.5g/dL). Activated partial thromboplastin time (APTT) was mildly elevated (mean 37.8s, SD 19.4), but mean prothrombin time (PT) and international normalised ration (INR) were within normal limits (PT 13.6s SD 3.4, INR 1.12, SD 0.35). Comparison of blood biochemistry, haematology and clotting results between mild and moderate/severe groups demonstrated significant difference in a number of variables. Haemoglobin, haematocrit, white blood cell count and platelet count were significantly lower in the moderate/severe group than in comparison to the mild severity group. APTT, INR, AST, ALT, CK and LDH were significantly higher in the moderate/severity group (Table 3.5, Figures 3.3, 3.4).

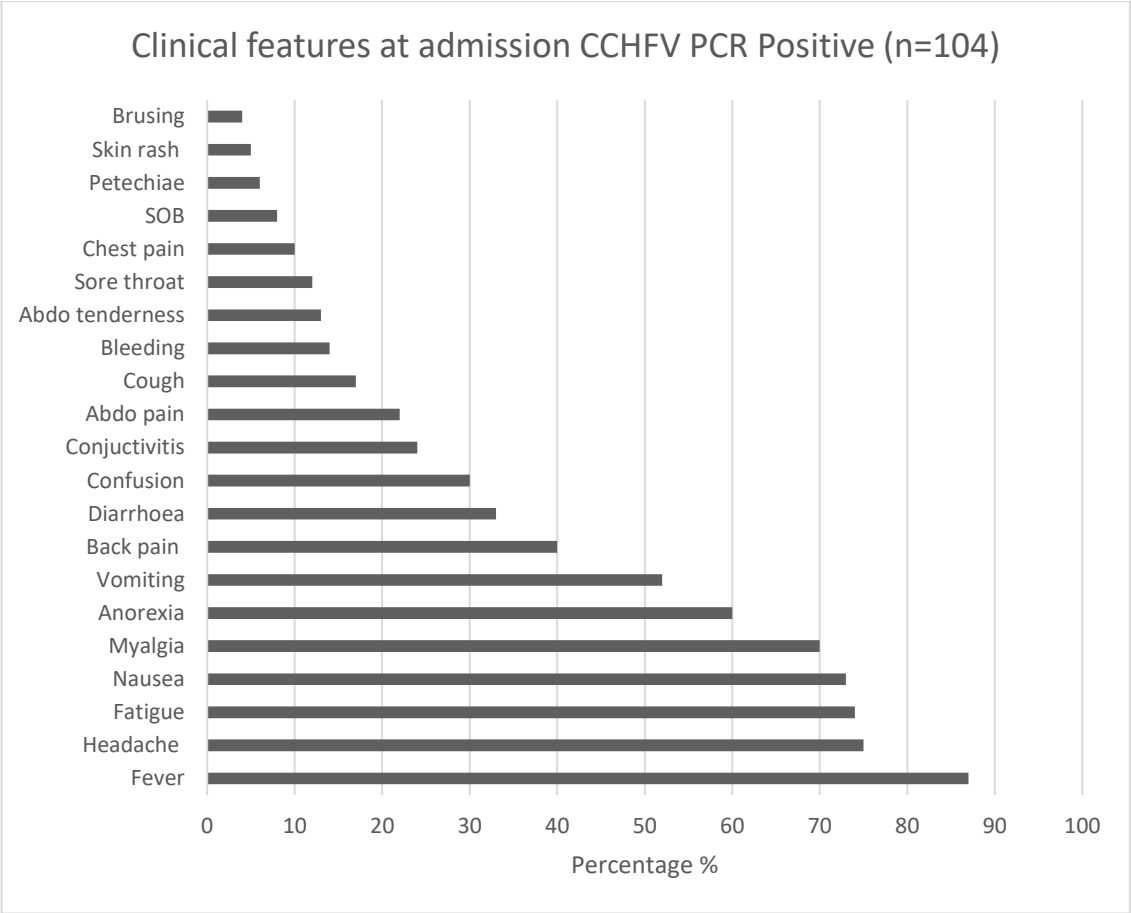


Figure 3.2 Bar chart showing frequency (%) of clinical features at admission of CCHFV PCR positive participants

Clinical Features at admission	Total (%)	Mild	Moderate/severe	p-value
Fever	90/103 (87.4)	56/63 (88.8)	34/40 (85.0)	0.56
Fatigue	77/104 (74.0)	51/63 (81.0)	26/41 (63.4)	0.066
Headache	79/104 (76.0)	49/63 (77.80	30/41 (73.2)	0.643
Myalgia	74/102 (72.5)	44/61 (72.1)	30/41 (73.2)	1.0
Anorexia	63/99 (63.6)	33/61 (54.1)	30/38 (79.5)	**0.018**
Swallowing Difficulties	23/103 (22.3)	13/62 (21.0)	10/41 (24.4)	0.637
Nausea	76/103 (73.8)	47/63 (74.6)	29/40 (72.5)	0.822
Vomiting	55/104 (52.9)	32/63 (50.8)	23/41 (56.1)	0.689
Diarrhoea	35/104 (33.7)	20/63 (31.7)	15/41 (36.6)	0.673
Abdominal pain	23/102 (22.5)	12/61 (19.7)	11/41 (26.8)	0.471
Hiccups	1/94 (1.1)	1/56 (1.8)	0/38 (0)	1.0
Shortness of breath	8/100 (8.0)	5/59 (8.5)	3/41 (7.3)	1.0
Chest pain	10/101 (9.9)	5/60 (8.3)	5/41 (12.2)	0.522
Sore throat	12/101 (11.9)	6/60 (10.0)	6/41 (14.6)	0.540
Conjunctivitis	25/101 (24.8)	18/60 (30.0)	7/41 (17.1)	0.164
Skin rash	5/102 (4.9)	2/61 (3.3)	3/41 (7.3)	0.389
Cough	18/101 (17.8)	11/61 (18.0)	7/40 (17.5)	1.0
Confusion	31/102 (30.4)	22/61 (36.1)	9/41 (22.0)	0.187
Abdominal tenderness	13/101 (12.9)	11/61 (18.0)	2/40 (5.0)	0.071
Hepatomegaly	2/78 (25.6)	0/48 (0)	2/30 (6.7)	0.171
Splenomegaly	4/78 (5.1)	0/49 (0)	4/29 (13.8)	**0.02**
Petechiae	6/103 (5.8)	0/62 (0)	6/41 (14.6)	**0.003**
Bruising	5/101 (5.0)	0/61 (0)	5/41 (12.2)	**0.009**
Bleeding	14/104 (13.5)	0/63 (0)	14/41 (34.1)	**<0.001**
Vital signs at admission				
Pulse -mean (SD) n=100	81 (8.3)	80.8 (6.5)	81.9 (10.5)	0.521
Systolic BP - mean (SD)	113 (13.3)	115.1 (14.1)	110.5 (11.4)	0.079
Diastolic BP – mean (SD)	69 (8.6)	70.4 (7.9)	67.7 (10.5)	0.132
Temp – mean (SD)	37.5 (0.85)	37.6 (0.8)	37.3 (0.9)	**0.035**
Respiratory rate – mean (SD) n=98	21.2 (2.4)	21.0 (2.1)	21.6 (2.7)	0.246
NEWS score at admission Median (SD) (98)	2 (0-8)	1 (0-5)	2 (0-8)	0.565

Table 3.4 Clinical features of CCHFV PCR positive participants at admission, stratified by disease severity. Categorical data analysed by Fisher's exact test and means compared by t-tests. Statistically significant results ($p<0.05$) highlighted in bold.

Blood results	Total	Mild	Moderate/ severe	p-value
Sodium (136-145 mmol/L) n=98	137.4 (3.6)	137.8 (2.9)	136.7(4.4)	0.121
Potassium (3.5-5.1 mmol/L) n=98	4.08 (0.44)	4.15 (0.36)	40. (0.53)	0.056
Blood urea nitrogen (BUN) (6-20mg/dL) median n= 94	22 (0.49 – 94)	23.5 (0.49-64.7)	17.6 (4.7-94.4)	**0.021**
Creatinine (0.7-1.2 mg/dL) median n=99	0.81 (0.4-4.6)	0.89 (0.4-2.6)	0.79 (0.41-4.6)	0.302
Glucose (74-109mg/dL) median N=88	110 (66-274)	112 (66-274)	107 (74-177)	0.290
Haemoglobin (14-18g/dL) n=103	13.5 (2.0)	13.9 (1.5)	13.0 (2.4)	**0.036**
Haematocrit (36-44%) n=102	40.2 (5.6)	41.3 (4.4)	38.5(6.8)	**0.014**
White blood cells (4-10 x 10^9/L) median n=103	2.74 (0.4-17.9)	2.85 (0.40-9.47)	1.8 (0.62-17.9)	**0.009**
Platelets (130-400 x 10^9/L) median n= 103	104 (6-231)	121 (36-231)	37 (6-119)	**<0.001**
APTT (25-37s) n=98	37.8 (19.4)	27.1 (9.3)	50.7 (24.5)	**<0.001**
PT (11-15s) n=96	13.6 (3.4)	13.2 (1.6)	14.2 (5.0)	0.167
INR (0.8-1.2) n=99	1.12 (0.35)	1.07 (0.15)	1.21 (0.52)	**0.045**
Amylase (28-100U/L) n=38	77.1 (36)	71.1 (34.4)	91.9 (36.9)	0.107
Bilirubin (0.1-1.2 mg/L) median n=74	0.39 (0.02-2.5)	0.36 (0.1-1.2)	0.44 (0.02-2.5)	0.106
AST (5-40 U/L) median n=101	79 (14-2779)	5 (14-172)	283 (19-2779)	**<0.001**
ALT (5-41 U/L) median n=101	44 (10-1055)	259 (10-103)	115 (15-1055)	**<0.001**
Creatine kinase (20-200 U/L) median n=80	298 (47-6355)	203 (47-3605)	413 (51-6355)	0.006
Fibrinogen (200-400mg/dL) n=15	291 (123)	333 (111)	245 (126)	0.179
LDH (135 -214 U/L) n=37	553 (205-3568)	359 (205-1068)	1147 (249-3568)	**0.001**

Table 3.5 Biochemistry, haematology and coagulation laboratory results at admission. Data are mean (SD), or median (range). Means were compared by t-tests and medians by Mann-Whitney U Test. Statistically significant results ($p<0.05$) highlighted in bold. APTT= activated partial thromboplastin time, PT= prothrombin time, INR= international normalised ratio, ALT = alanine transaminase, AST= aspartate transaminase, LDH= lactate dehydrogenase.

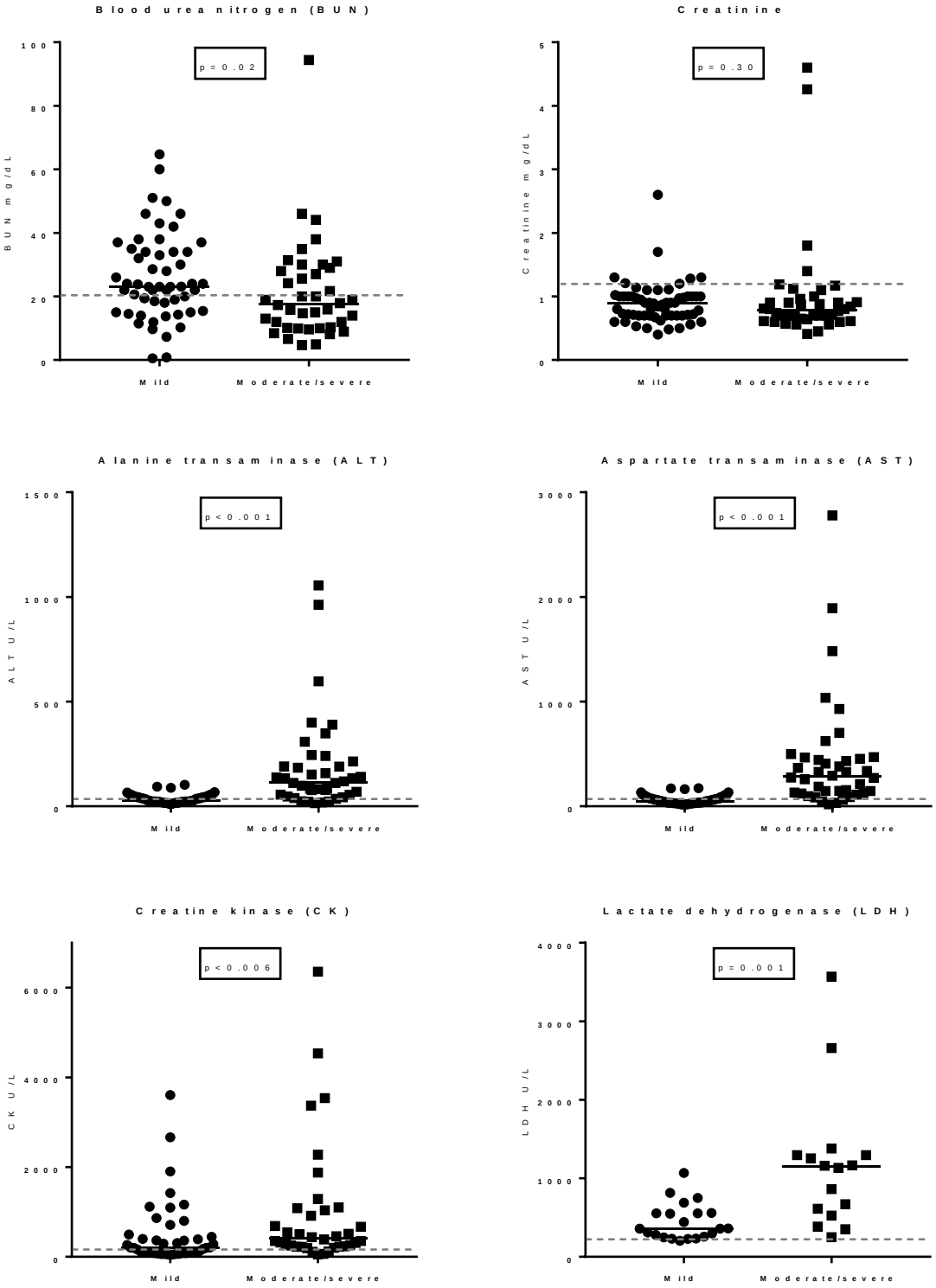


Figure 3.3 Biochemistry laboratory results at admission by severity group. Scatter dot plots with median displayed and upper limit normal range (dotted line). p values are from Mann-Whitney U Tests.

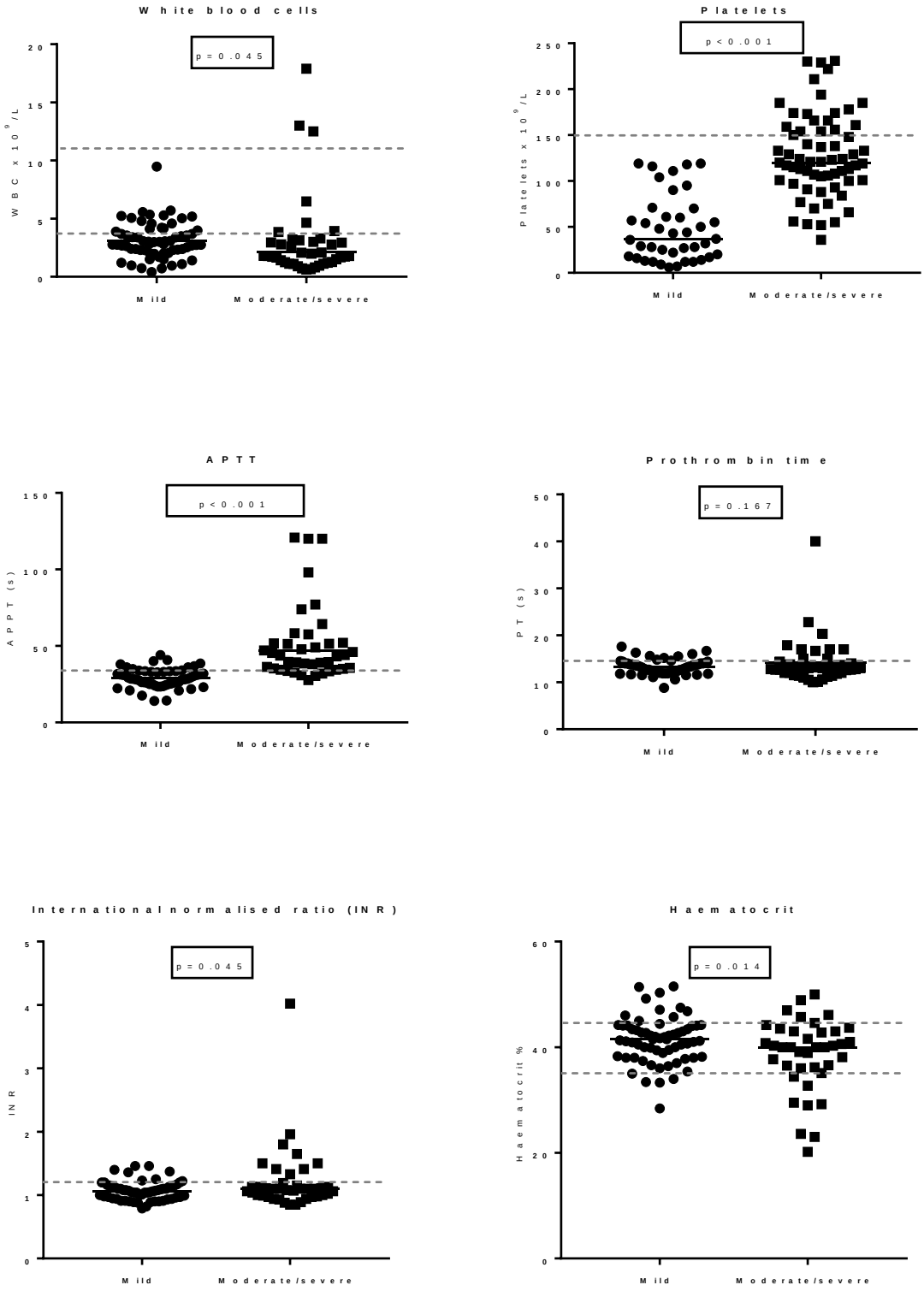


Figure 3.4 Haematology and coagulation laboratory results at admission by severity group. Scatter dot plots with median/mean displayed and upper/lower limit normal range as appropriate. p values are from t tests (white blood count and platelets) and Mann-Whitney U Tests (all others).

3.2.2 Clinical features during hospitalisation

During the course of hospital admission patients were assessed daily to characterise the natural history of confirmed CCHF disease. Bleeding occurred in 25/104 (24.0%) participants for a median of 2 days duration (IQR 2-5 days). Headache was reported by 72/104 (69.2%) participants for a median of 3 days duration (IQR 2-5 days). Myalgia was reported by 66/104 (63.5%) participants for a median 3 days duration (IQR 2-5 days). Diarrhoea occurred in 41/104 (39.4%) and vomiting in 70/103 (67.3%) participants, lasting a median 2 days (IQR 2-3 days) and 2 days (IQR 1-3 days) respectively. Lethargy was reported by 75/104 (72.1%) lasting a median 5 days (IQR 2-6 days).

Vital signs (temperature, respiratory rate, heart rate, systolic and diastolic blood pressure) were recorded daily for a total of 749 patient days for 103 participants. For each vital sign, the most abnormal reading within that 24hr period was recorded. Fever ($\geq 38^0$C) occurred in 54/104 participants during the course of hospital admission lasting for a median 2 days (IQR 1-10 days). The number of days of fever was significantly shorter in moderate/severe group than in the mild group (0 days (IQR 0-1 days) vs 1-day (IQR 0-2 days) p=0.005).

Trends in vital signs during acute infection were analysed by simple linear regression analysis and stratified by CCHF severity. There was no significant trend for blood pressure (p=0.66) and respiratory rate (p=0.182), but significant slopes for temperature (p<0.0001) and heart rate (p=0.02), with both reducing as illness progressed. When blood pressure was stratified by disease severity, there was no difference in slopes, but lower elevations/intercepts in BP in moderate/severe disease (p=0.0003), compared to mild disease. Respiratory rate stratified by disease severity conversely had a higher elevation/intercept in moderate/severe disease (p<0.0001).

Linear regression of temperature by severity demonstrated significant non-zero slopes for both mild (p<0.0001) and moderate/severe disease (p<0.0001), with unequal slopes (p=0.0003). Heart rate stratified by disease severity showed no significant trends (mild p=0.31, moderate/severe p=0.07), and no difference in slopes (p=0.17) or intercept/elevations (p=0.35) (Figure 3.5).

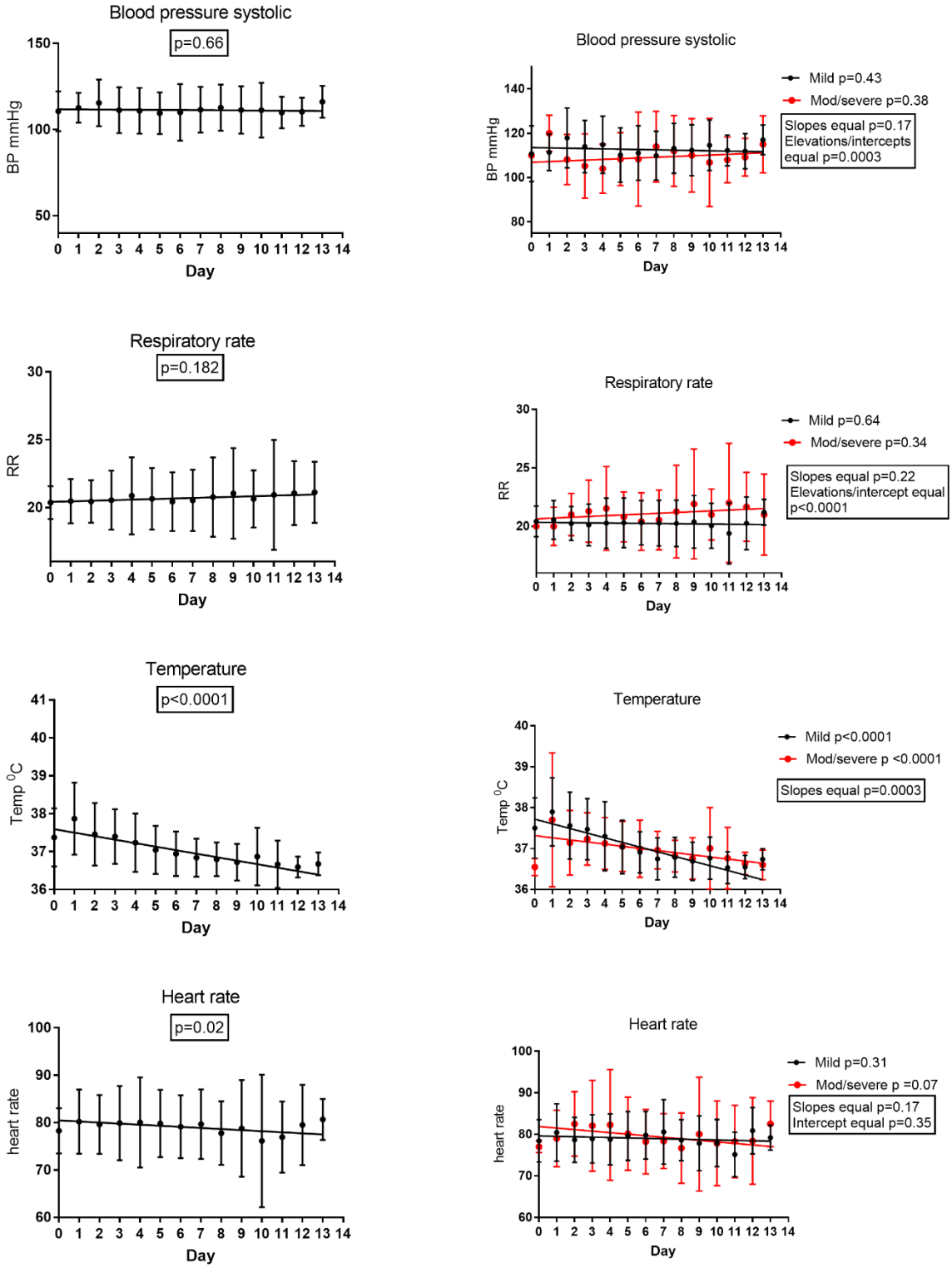


Figure 3.5 Longitudinal vital signs by day of illness. Mean and SD are plotted with linear regression lines. p-values indicate if slopes are significantly non-zero. When stratified by CCHF severity p-values indicated if slopes or intercepts/elevations are equal.

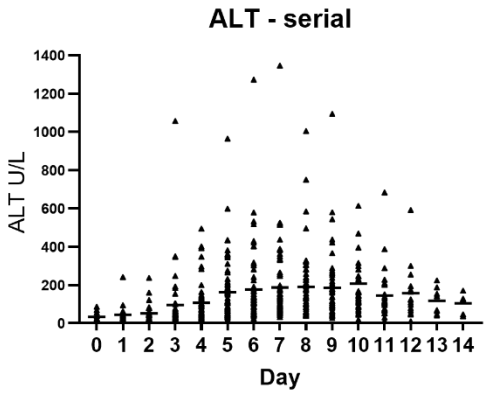
3.2.3 Laboratory results during hospitalisation

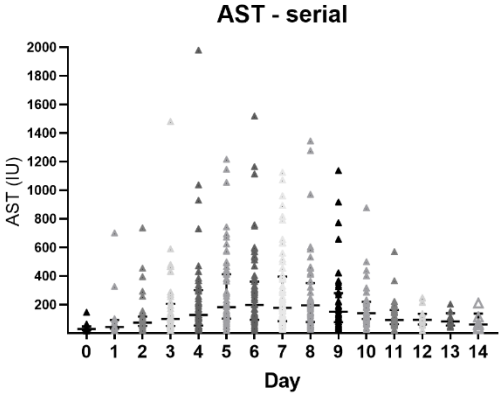
During the study period participants had daily phlebotomy for both routine and research samples. Routine haematology, coagulation and clinical biochemistry samples were analysed in study site laboratories with individual clinicians deciding on the frequency of tests. The total number of tests in the CCHF PCR positive cohort (104 participants) were: Haematology parameters (WBC 782, Plts 779, HCT 782); Coagulation (APTT 494, PT 483), Biochemistry (CK 454, Creatinine 431, AST 561, ALT 562). The serial daily blood results of these parameters are demonstrated in Figure 3.6.

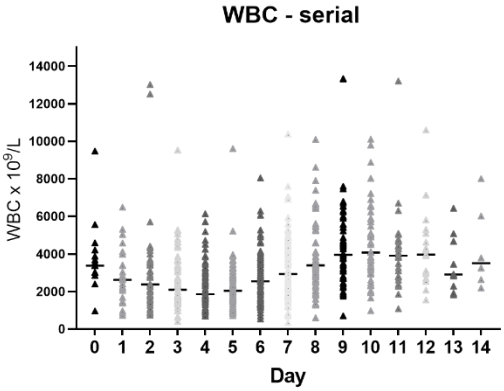
Alanine transaminase and aspartate transaminase (ALT/AST) reach peak levels at day 7/8 of illness. The lowest median white blood count was at day 4 with the median lowest platelet count occurring at day 6 of illness. There were only mild elevations of direct and indirect bilirubin. The highest median/peak of creatine kinase observed was at Day 4 whilst there was no clear patterns for creatinine, prothrombin time or haematocrit. Activated partial thromboplastin time was highest at day 4.

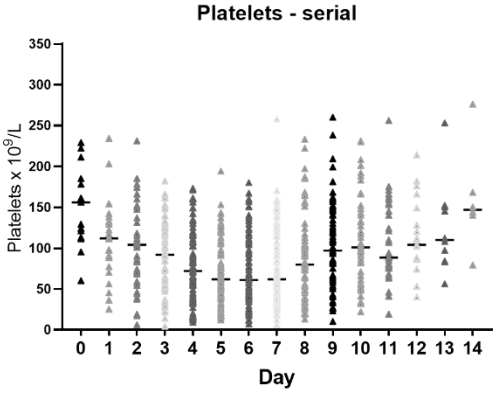
Acute renal impairment occurred in 4/104 patients during the study period. By RIFLE criteria 1/4 had RIFLE stage 1 (risk – creatinine 1.5 x upper limit normal (ULN)), that was present at admission and then improved during admission. One of the 4 patients had RIFLE stage 2 (Injury – 2 x creatinine ULN) that responded to fluids and improved, and 2 patients had RIFLE stage 3 (Failure – 3 x creatinine ULN) that was associated with a fatal outcome within 24hrs, one of which received haemodialysis (Figure 3.8). At the time of the AKI stage 3 creatinine kinase levels were elevated at 688/685 U/L.

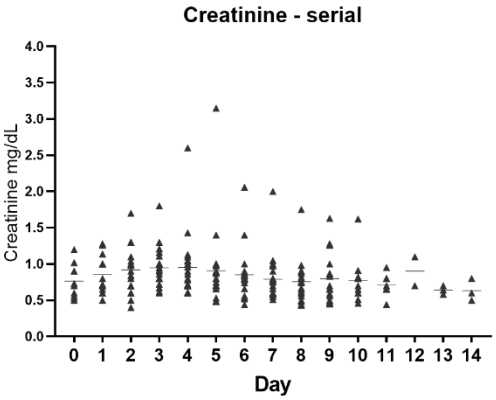
Stratification of laboratory results by CCHF severity groups is shown in Figure 3.7. AST and ALT were higher in the moderate/severe group during acute illness until day 10/11. Conversely platelet count is lower in the moderate/severe group until day 11/12 with no clear differences shown in white blood cells, creatinine and creatine kinase during acute illness between severity groups. Median APTT is higher in the moderate/severe group until day 7/8 of illness, with PT higher in the first 0-2 days of illness in the severe/moderate groups. Median haematocrit levels were lower in the moderate/severe group during acute illness.

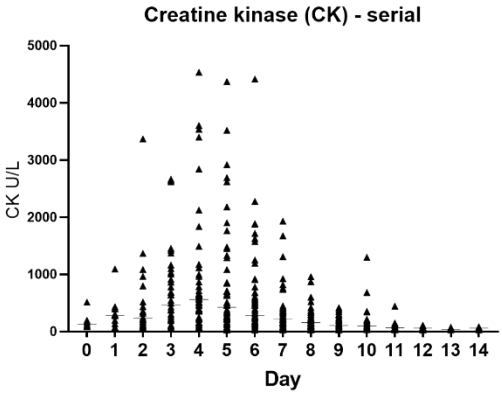

ALT - serial
ALT U/L
Day


AST - serial
AST (IU)
Day


WBC - serial
WBC x 10^9/L
Day


Platelets - serial
Platelets x 10^9/L
Day


Creatinine - serial
Creatinine mg/dL
Day


Creatine kinase (CK) - serial
CK U/L
Day

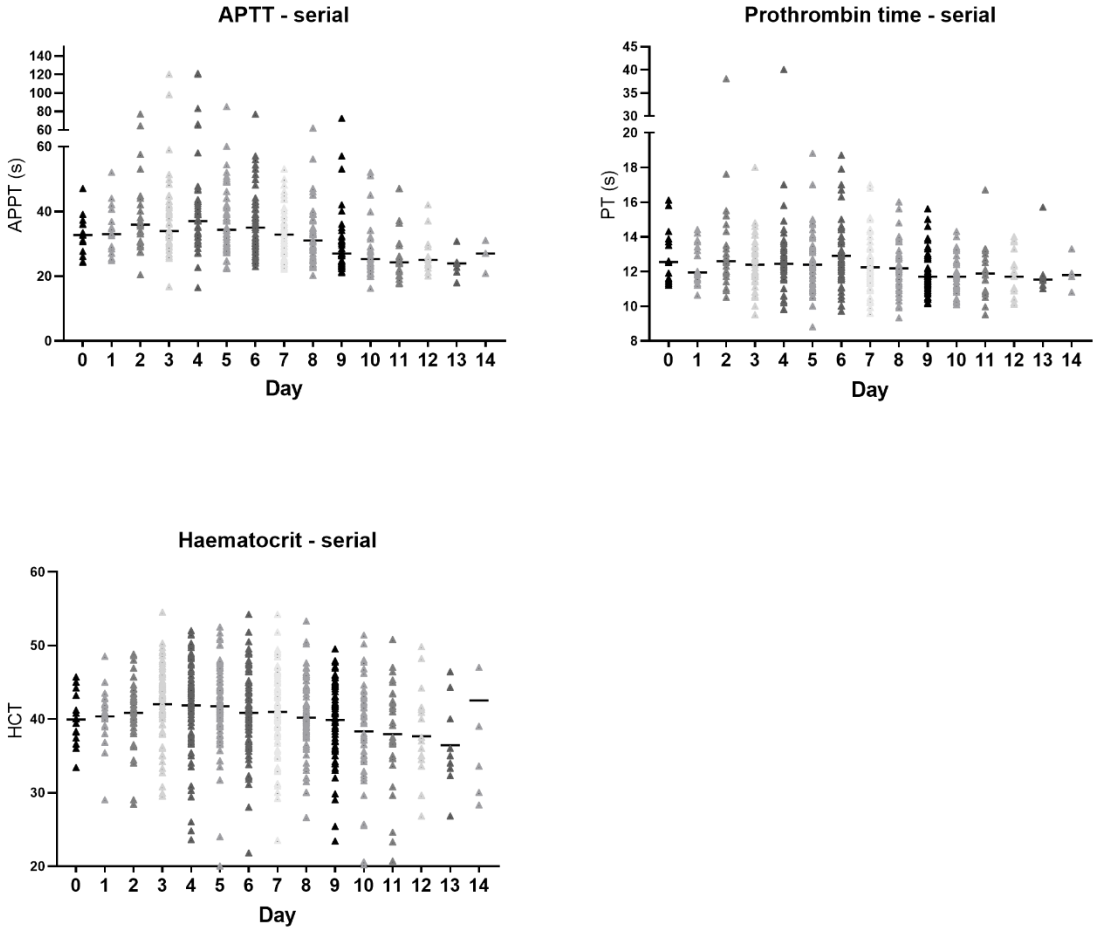
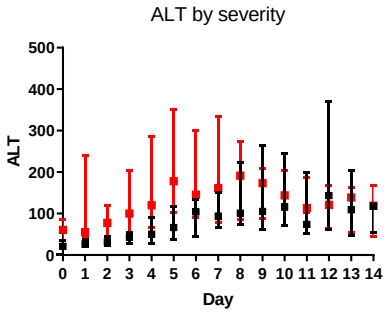
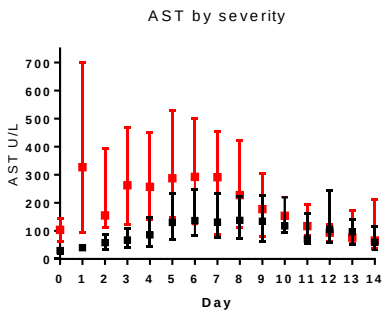
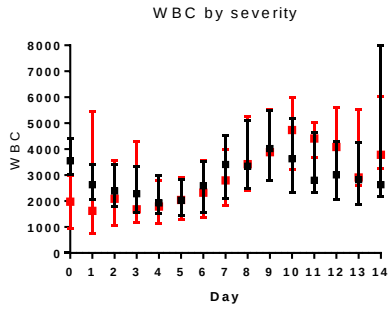
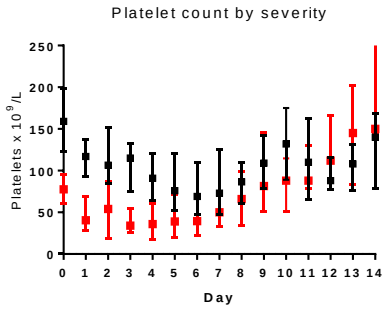


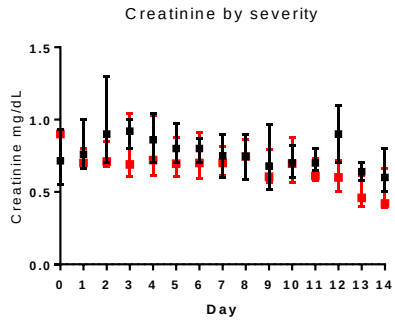
Figure 3.6 Longitudinal aligned dot plot of haematology, biochemistry and coagulation laboratory variables during acute admission by day of illness. Lines indicate median values.

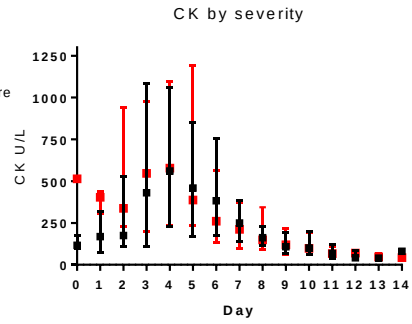

ALT by severity
ALT
Day
Mild
Moderate/severe


AST by severity
AST U/L
Day
Mild
Moderate/severe


WBC by severity
WBC
Day
Mild
Moderate/severe


Platelet count by severity
Platelets x 10^9/L
Day
Mild
Moderate/severe


Creatinine by severity
Creatinine mg/dL
Day
Mild
Moderate/severe


CK by severity
CK U/L
Day
Mild
Moderate/severe

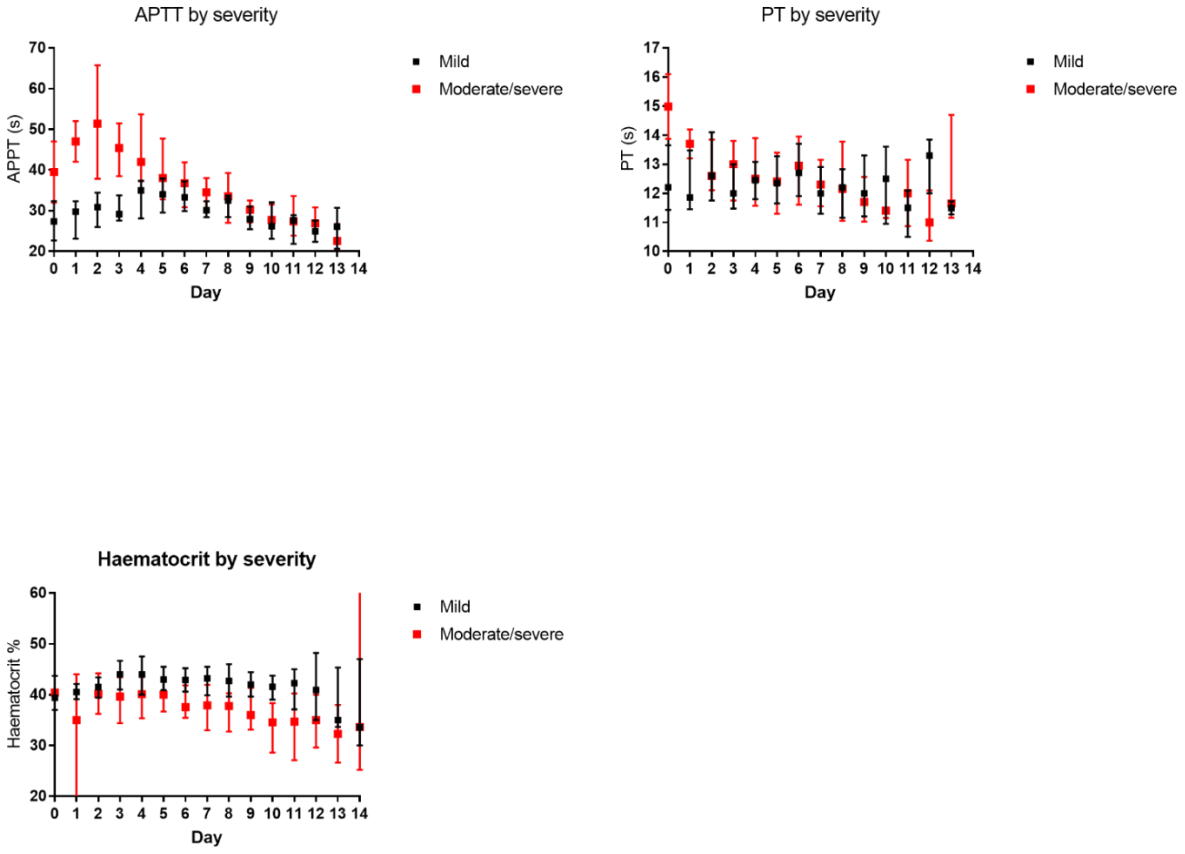


Figure 3.7 Serial haematology, biochemistry and coagulation laboratory parameters during acute admission grouped by CCHF disease severity. Median and IQR displayed for mild and moderate/severe groups by day of illness.

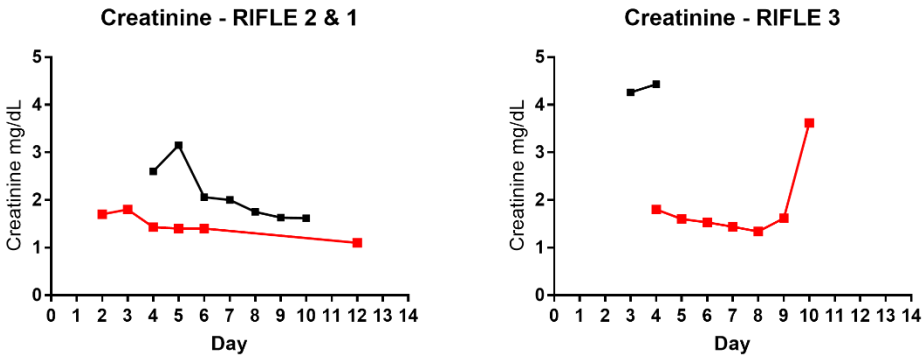


Figure 3.8 Serial creatinine levels by day of illness in participants with RIFLE stage 1&2 (n=2) and RIFLE stage 3 (n=2) acute kidney injury

3.3 Treatment received during hospitalisation

All participants were admitted to a trial site/hospital during the study and all (104/104) had an intravenous catheter placed. Intravenous fluids were administered to 90/104 participants for a median 7 days (IQR 5-9 days). 2/104 patients were admitted critical care units with severe CCHF during hospitalisation and received ventilatory and vasopressor support for 1 day prior to death from multi-organ failure and septic shock. Oxygen supplementation was only administered to the 3 fatal cases.

Blood components (platelet, fresh frozen plasma and packed red blood cells) were given to 38/104 participants. In the mild group 11/63 (17.5%) of patients received blood products compared to 27/41(65.9%) in the moderate/severe group ($p<0.0001$). Platelets were the most frequent blood product utilised, received by 37/104 (35.6%)participants (mild 11/63 vs moderate/severe 26/41, $p<0.0001$). The median units of apheresed platelets received was 4 units (IQR 2-7 units). Fresh frozen plasma (FFP) was administered to 25/104 (24.0%) participants (mild 5/63 vs moderate/severe 20/41, $p<0.0001$), with a median of 4 units given (IQR 2-11 units). Packed red blood cells were less frequently administered, and 12/104 (11.5%) participants (mild 9/41 vs moderate/severe 3/63, $p=0.01$) received a median of 2 units (IQR 1-3 units). Of the patients that received FFP, only 8/25 had either an APPT or PT >1.5 times the upper limit of normal. A further 8/25 had PT/APPT parameters less than this but had clinical evidence of bleeding and a 1/3 had neither bleeding or low PT/APTT levels. Platelet transfusion was administered to 37 participants of which 36/37 had a nadir platelet count $<50 \times 10^9$/L and 22/37 had a nadir platelet count $<20 \times 10^9$/L. This represented 36/56 patients with platelet counts $<50 \times 10^9$/L receiving platelet transfusion, with all patients with platelet counts $<20 \times 10^9$/L receiving platelet transfusion.

3.3.1 Ribavirin treatment

Ribavirin was administered to 36/104 participants, all of whom were managed at one study site after direct admission or hospital transfer. Ribavirin treatment was started a median of 4 days (IQR 2-5 days) after onset of symptoms. Ribavirin was administered orally for a median of 5 days (IQR 4-8 days), at standard dosing (2g loading day 1, 1g QDS days 1-4 and 0.5g QDS days 5-10). At the time that ribavirin was initiated 25/33 (3/36 no matching PCR sample) patients were still CCHFV positive, with 8/33 having already cleared CCHFV from blood. Ribavirin was administered for a total of 201 patient days in participants with matched daily CCHFV blood PCR results. For 144 of the 201 days of ribavirin administration participants were CCHFV PCR negative. Comparison between treatment groups showed that 16/36 (44.4%) patients that received ribavirin were graded as moderate/severe compared to 25/68 (33.7%) graded moderate/severe in the non-ribavirin group.

3.4 Discussion

The planned sample size of 100 PCR positive patients was achieved from the 3 study sites in Samsun and Tokat. During planning for the project, it was recognised that participants had to be recruited at the suspected CCHF stage to allow for early sampling for viral dynamics and host immune response investigation. There was also the chance that patients would rapidly deteriorate and lose the capacity for informed consent. During the course of recruitment only 4 eligible patients refused consent to enter into the study and 2 subsequently refused further sampling and attendance at follow-up visits. CCHF PCR results were generally available 24-48hrs after admission and 104/144 patients recruited were PCR positive and continued in the study.

The demographics of the cohort were similar to larger published epidemiological studies in Turkey, such as by Yilmaz et al that reported epidemiological data on 1820 patients from 2002-2007 [141]. A slightly higher percentage of male patients were recruited (63.5%), and the median age was higher than expected (50 years, Yilmaz et al [141] 44 years). There was only limited past medical history/comorbidities identified in the cohort, with diabetes and cardiovascular disease the most frequent. Comorbidity was not overrepresented in those with more severe CCHF.

The majority of patients recruited had a history of tick bite (70%), consistent with data across Turkey [15], with symptoms developing a median of 3 days after the tick bite. Patients generally presented rapidly to hospital (median 3 days), with only 3 participants being admitted after 7 days of illness. The vast majority of participants lived in CCHF endemic areas (91%) and 82% had close animal contact with around 50% also having direct contact with animal blood or tissue, both higher than Yilmaz' report (32% animal contact and 10% blood or tissue contact). There was a low rate of contact with others with similar illnesses (3%), as reported by Yilmaz et al [141], and this provides further evidence that community based person to person transmission is rare. No contacts of any of the 104 were admitted or tested for CCHF during the study.

There are three published CCHF severity scoring systems that utilise a combination of clinical and laboratory factors, although none include the crucial parameter of CCHF viral load. The oldest was developed by Swanepoel and relies on 5 parameters during the first 5 days of illness aimed at predicting mortality. Two further scoring systems have been developed and validated by investigators in Turkey. They both use a combination of clinical and laboratory parameters to group patients into low, intermediate and high risk (severity grading system) and mild, moderate and severe (severity scoring index).

The SSI has the advantage of having only 5 parameters and is based on admission data but does include fibrinogen level which is not routinely available in many laboratories in endemic settings. Based upon a retrospective validation of the SSI in 281 patients, they report a case

fatality rate of >50% in severe cases (score >10), and that a high negative predictive value at admission demonstrated that mild cases (score 0-2) would not progress to death[154]. The SGS utilises 12 parameters in the first 5 days of illness but includes variables such as lactate dehydrogenase and separate parameters for INR and prothrombin time. Bakir et al[153] prospectively evaluated the SGS in multiple centres in Turkey in 2012 showing that all low risk (score 0-4) cases survived (n=323), those in the intermediate group (score 4-8) had a case fatality rate of 20% (14/70) and in the high risk group (score >9) all patients died (9/9).

Although both the SSI and SGS scoring systems have been evaluated in large cohorts in Turkey they are not in routine use in most specialist CCHF centres. The ability to predict mortality is useful for healthcare workers, especially in endemic setting when managing large cohorts, but better predictive value is required for other key outcomes such as blood product requirements, critical care interventions and length of stay. Severity scoring systems would also be useful if they were able to also predict the risk of viral shedding/nosocomial infection and as such level of personal protective equipment required. Above all they need to be simple and based on widely available clinical and laboratory factors if they are to be in routine use. Currently in Turkey the most frequently used parameter to guide patient treatment and whether they require transfer to specialist tertiary units is a platelet count $<50 \times 10^9$ /L. Other factors such as age, bleeding and somnolence are also recognised as risk factors and influence patient management and disposal [180].

In this cohort we decided to score all participants utilising all 3 scoring systems at admission/first 5 days of illness and then categorize them as 'mild' if they had none of the Swanepoel parameters, and were graded as mild and low risk by SSI and SGS respectively. The 'moderate/severe group' included all patients graded as moderate/severe (SSI), intermediate/high risk (SGS) or with Swanepoel criteria. The SSI system categorised the majority of participants in the moderate/severe group (39/41), with the SGS conversely recording more patients as mild. Of note all three fatal case were recorded as moderate by SSI, had Swanepoel criteria and by SGS 2/3 were intermediate risk and 1/3 graded high risk. A newer simpler CCHF severity score is required, probably incorporating CCHF viral load and other simple parameters such as platelet count and bleeding.

The clinical features of the participants at admission is also consistent with larger data sets, although bleeding was less frequent (14%) than that reported by Yilmaz et al[141] (23%) and Bakir et al[152] (18%), and this probably reflects the higher proportion of patients were directly recruited from Tokat State hospital, with early/mild disease. There is limited data on vital signs at admission in patients with CCHF. The vast majority were normal at admission, with median NEWS scores of 2 and 1 in moderate/severe and mild disease groups respectively. Interestingly

although a history of fever is reported by 90% of patients with CCHF the mean temperature was 37.5^{0}C. Although arbitrary temperature cut offs are frequently used in case definitions for surveillance and screening, only 37% of this cohort had a temperature of ≥38 ^{0}C, and 48% a temperature of ≥37.5 ^{0}C at admission. This is a key translational finding of the study with clear policy implications and is consistent with data on Lassa fever from Nigeria [94] and from the Ebola virus disease outbreak in West Africa [181].

Clinical and laboratory features at admission are important for diagnosis, surveillance and incorporating into severity scores, but limited data exists on the natural history/evolution of CCHF and other viral haemorrhagic fevers. We collected daily routine laboratory variables, and also the clinical features of CCHF in the cohort through a standardised case record form. The most frequent symptoms reported were headache, myalgia, lethargy and vomiting, all occurring in approximately 70% of patients for 2-3 days. In total 23% of the cohort had an episode of bleeding, that lasted for a median 2 days. During the course of admission fever above 38^{0}C was only recorded in 50% of patients, and the duration of fever was surprisingly shorter in the moderate/severe group compared to the mild group, with different slopes demonstrated in linear analysis. Heart rate also reduced during the course of illness, and as might be expected the moderate/severe group had a lower systolic blood pressure and higher respiratory rate elevation/intercepts. Review of the vital sign data for the cohort demonstrates that in the majority of cases, a significant systemic inflammatory/sepsis response is not evident. It is also important to highlight that whilst approximately 25% of patients had bleeding, most patients did not demonstrate evidence of disseminated intravascular coagulation or multiorgan failure. This is in contrast to what has been observed in patients with EVD [182,183], and also Lassa Fever [94,168,184] where haemorrhage occurs late and is a poor prognostic marker.

Some of the serial laboratory data showed clear patterns. The peak elevations of liver enzymes occurred later in the disease course than that observed for CK and APTT. The lowest white cell counts were observed 2 days earlier than the lowest platelet counts on days 5-6. This provides insights into disease pathogenesis, and whilst leucopenia is common in many acute viral infections, this may highlight CCHFV potentially disrupting cells in the bone marrow resulting in decreased production of leucocyte precursors, direct infection and bystander apoptosis[86,185,186].

The rise in liver enzymes probably represent dissemination of infection and then viral replication within hepatocytes, that have been demonstrated in animal models [88] and suggests the liver may have a significant role in amplifying CCHFV. Marked histopathological changes have also been reported showing a cytopathic effect, with hepatocellular necrosis varying from

mild to severe, with focal area marked by haemorrhage and associated with the eosinophilic change of hepatocytes and Councilman bodies. The degree of necrosis has also been shown to correlate with the degree of elevation of liver enzymes [187], and although not seen in this study fatal cases of liver failure occurring in CCHF have been reported [157,188]. In this study both ALT and AST were higher in the moderate/severe group as might be expected. However, 10% of the mild group went on to develop AST levels greater 500IU/L during the course of admission, illustrating the difficulties of categorisation at admission.

Thrombocytopenia is a consistent feature reported in CCHF, and has been shown to be a simple predictor of mortality [150,151,189,190]. A number of mechanisms that lead to the thrombocytopenia seen in CCHF have been suggested including due to decreased production due to bone marrow hypoplasia, endothelial damage stimulating platelet aggregation and finally increased platelet consumption as a result of DIC [90,191,192]. However previous hypotheses in CCHF, and viral haemorrhagic fevers in general have been based on traditional views that the cellular activities of platelets are restricted to rapid pro-coagulant responses that lead to platelet aggregation and granule secretion. However, as well as their key roles in maintaining haemostasis, platelets are now increasingly recognised as major inflammatory cells with roles in both the innate and adaptive immune systems [193]. Platelets have been shown to express surface receptors able to mediate binding and entry of a range of viruses [194,195] and it is now known that they express various pattern recognition receptors (PRR) that respond to a range of infecting microorganisms including viruses. Although they do not have nuclei, they have all the molecular machinery that may enable RNA viruses to synthesize proteins such as cytokines [193].

Dengue infection is the commonest viral haemorrhagic fever that has a spectrum of clinical illness and thrombocytopenia has been shown to correspond to disease severity. Whilst the pathophysiology of severe dengue syndromes is not fully understood, a pro-inflammatory response, similar to that reported in CCHF, and platelet activation have been associated with disease severity [196,197]. The viral genome of dengue virus (a Flavivirus) and endocytosed dengue virus like particles have also been detected in circulating platelets from infected patients [198,199] . There is also evidence of viral replication and translation of the viral genome , but whether infectious virus is generated is unclear [200].

In dengue infection it remains unknown whether viral attachment or internalisation is required for platelet activation [193]. This may then contribute to platelet loss by mediating platelet deposition in microvascular bed and through clearance of activated platelets by inflammatory monocytes [200]. Interestingly, it has also been demonstrated that platelet activation is maximal when virus particles are no longer circulating , indicating that additional mechanism such as pro-inflammatory mediators drive platelet activation after clearance of viraemia [185,201], consistent

with maximal thrombocytopenia being seen later in CCHF. Increased platelet apoptosis is also thought to contribute to the thrombocytopenia in dengue, due to activation of an intrinsic death program involving apoptotic caspases, that has also been linked to disease severity [185,201].

Renal dysfunction was not commonly seen in this cohort of CCHF with only 4/104 having acute kidney injury by RIFLE criteria. There are two cases of acute renal failure in the study, that has been reported previously in CCHF [139,159] although the mechanism of action remains unclear. Although creatine kinase levels are elevated in the patients with acute renal failure, suggesting a degree of viral myositis/rhabdomyolysis they are not at the levels that would be associated with significant renal impairment. A case report of a patient in Iran with suspected thrombotic microangiopathy and acute renal failure, that was subsequently confirmed to have CCHF, had a renal biopsy that revealed normal looking glomeruli and renal vasculature. Intrarenal hemodynamic dysregulation secondary to cytokines and endothelial dysfunction mediated by cytokines or virus were suggested as possible explanations [159]. In a study of 44 children in Turkey with CCHF with normal renal function, proteinuria was found in 60% and increased uNGAL levels, an early and sensitive marker of AKI, found in severe patients [202]. Although rare, the aetiology of renal dysfunction requires further evaluation to understand how much is a result of either direct viral renal damage, renal hypovolemia due to sepsis or as a consequence of DIC.

Supportive care of patients during the study was guided by infectious diseases physicians in the different sites, with the vast majority (>98%) receiving ward level care. Intravenous fluid supplementation was administered to the majority of participants, for the entirety of their medical admission: this established practice was not generally guided by fluid losses or evidence of hypovolaemia/renal dysfunction. Only 2 patients received additional critical care support, both in the final stages of illness and lasting less than 24 hours. This further demonstrates the high percentage of 'mild' CCHF seen in Turkey and recruited in the study that is different to anecdotal clinical experience in Kosovo or South Africa.

Blood product replacement is a key intervention in CCHF, aimed at managing the coagulopathy and preventing haemorrhage. Receiving blood products was clearly linked with the severity grouping for all blood components, although this has not been reported before when the scoring systems were independently validated. However, bloods products were utilised in patients that were graded as mild/low risk by all severity systems, recognising limitations of severity scores to predict the need for transfusion. More important was the decision making around use of blood products by clinicians and whether this could be rationalised and protocolised. Of the patients that received FFP, only 1/3 had either an APPT or PT >1.5 times the upper limit of normal. A further 1/3 had PT/APPT parameters less than this but bleeding and a

1/3 had neither bleeding or elevated PT/APTT levels. Platelet transfusion was generally administered according to absolute platelet count, with 36/37 receiving transfusion having platelet counts <50 x 10^9/L and all patients with platelet counts <20 x 10^9/L receiving platelet transfusion.

The main limitations in the clinical and laboratory description of the natural history of CCHF are that a smaller number of fatal and severe disease were recruited than planned. This limits the ability to improve understanding of the more complicated spectrum of CCHF disease, but unlike other reports does describe a large cohort of 'mild' disease for comparison. The other main limitation was inconsistent or missing laboratory data due to daily laboratory tests being determined by individual clinicians. At the design stage of the study this practice was evaluated and the case record form piloted in one centre (OMUH) that undertook most biochemistry, coagulation and haematology assays daily, in line with recently published recommendations [160]. As the study extended to other sites, regular assessment of coagulation and biochemistry parameters became less frequent and was guided by the clinical picture, which whilst clinically appropriate provided less data to inform understanding of the natural history of disease. A detailed schedule of the minimum frequency of laboratory analysis would have mitigated this, but it must also be recognised that this would have sent additional samples containing CCHFV to standard laboratories, for research as opposed to direct patient care need.

In summary, the epidemiological, clinical and laboratory data is broadly consistent with published data sets in Turkey, apart from a significant proportion of 'mild' disease severity being recruited. This is evidenced by the low mortality, severity scoring approach and lower rates of key outcomes such as bleeding and critical care admission. The first description of the use of early warning scores further demonstrates this and provides the first evidence that the pattern and aetiology of most bleeding that is seen is not due multi-organ failure/DIC , supported by normal observations and other laboratory parameters at the time. As has been shown in other VHFs, only a third of patients at admission have temperatures >38^{0}C, that has important implications for case definitions and surveillance. Simultaneous evaluation of the scoring systems in parallel has further demonstrated that additional variables, and simplification for routine use is required.

Longitudinal data highlighting the natural history of CCHF is lacking and this data set provides detailed clinical and laboratory data highlighting the difference by disease severity and over time. Thrombocytopenia is the key laboratory variable that in practice guides patient disposal and management in the Turkey, but the pathogenesis remains poorly understood and may be multifactorial like suggested in dengue infection. Case management in Turkey is known to be of a high standard, but improved utilisation of blood component therapy, potentially supported by

novel platforms such as ROTEM as discussed later may rationalise and improve this approach. Ribavirin therapy was utilised in a third of participants, generally relatively early in disease course (median day 4), and in more severe disease groups. In most cases in Turkey it is administered orally, with reduced bioavailability [203] that may limit its effect. Its relationship to CCHF viral load is evaluated further in the next chapter.

Chapter 4 CCHF Viral Load

4.1 Introduction

The viral load, when measured in diagnostic blood samples at admission has been shown to be an important prognostic indicator in Ebola Virus Disease [93,204] and Lassa Fever [94]. A similar correlation between viral load at presentation and disease severity/outcome in CCHF has also been demonstrated in a number of studies. The first CCHF VL data were published by Cevik et al [95] in 36 patients from Turkey and Duh et al [96] from 24 patients in Kosovo in 2007, demonstrating that CCHF viral loads were higher in fatal than surviving cases. In both studies a VL greater than 1×10^9 copies/ml was indicative of a fatal outcome.

This has been confirmed by a number of further studies [97–99], some of which also found associations between viral load and higher disease severity scores. A number of studies also investigated viral loads serially with daily sampling, the largest of which by Bodur et al [100], measured viral loads daily in 50 patients. By day 3 of admission 50% of patients had cleared virus, with only 1 /42 having a positive PCR on day 6. This is consistent with Cevik et al's [95] first report and more recent data [101] demonstrating that virtually all patients had cleared viraemia within 6 days of admission, with patients being admitted 3-5 days after onset of symptoms. There are limited data on the viral dynamics of CCHFV in urine. This could have important diagnostic implications if CCHFV is detectable in urine after clearance from the blood and potential transmission risks. Prolonged viraemia in urine (>30 days post disease onset) has been demonstrated in one report from Kosovo [103], but this hasn't been evaluated in other large cohorts.

4.2 Methods

Patients with suspected CCHF were admitted to the study hospitals and had blood samples sent to the Regional PH reference laboratory in Samsun for CCHF PCR. After recruitment into the study patients had daily blood sampling during acute admission. EDTA blood samples were centrifuged, plasma aliquoted in a Class 1 BSC and stored at -70^0C in the study laboratories in Samsun and Tokat. Urine samples were also collected daily and stored at -70^0C. Urine and plasma samples were then transferred on dry ice with temperature monitoring to PHE Porton for CCHF PCR. Serum samples were analysed by ELISA for CCHF IgM/IgG at PHE Porton. Data was analysed according to statistical methods described in chapter 2.

PHE Porton RNA extraction and PCR

1. One hundred forty microliters of supernatant were added to 560 µL AVL buffer for RNA extraction in sealable deep 96-well plates for removal from the CL4 laboratory.
2. Extraction of RNA was performed using the MagnaPure 96 small volume RNA kit (Roche, Burgess Hill, UK), a magnetic bead-based method of RNA separation.

3. Samples were vessel transferred from plates taken from containment level 4 (CL4) into MagnaPure plates at CL3 prior to loading onto the MagnaPure 96 automated extraction robot and RNA eluted in 60 µL nuclease-free water.
4. Target amplification was performed using an internal PHE assay with primers to the Crimean-Congo haemorrhagic fever glycoprotein as described in Atkinson et al 2012 [205] using the Fast Virus qRT-PCR Kit (Qiagen, Manchester, UK). The assay is designed based on an untranslated region of the CCHF S segment between pan-handle loop structures and has been validated for diagnostic use by the Rare & Imported Pathogens Laboratory, Public Health England.
5. Analysis was performed using the ABi 7500 (Applied Biosystems, Paisley, UK) under the following cycling conditions: 50 °C for 10 min, 95 °C for 30 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 3 s; temperature cycling was set to the maximum ramp speed, and data were acquired and analysed using the ABi 7500 on-board software version 2.0.6 (Applied Biosystems, Paisley, UK) with a threshold set to 0.05.
6. Cycle threshold (Ct) values from the PCR assay were used to give a consistent reading of the amount of RNA levels in the samples. To calculate patient viral load Ct values were compared with a standard curve of synthetically produced RNA fragments (GeneArt) encoding the target region in the S segment of CCHFV.

4.3 Results

Of the 144 participants recruited with suspected CCHF, 818 plasma samples were collected and analysed for CCHFV by PCR. 569 acute samples taken during hospital admission were analysed from the 104 participants with PCR confirmed CCHF infection (at Samsun PH reference laboratory), of which 144/569 were CCHFV PCR positive in PHE Porton.

In total 56/104 participants that tested PCR positive in Turkey, had samples taken that were also PCR positive in PHE Porton. Participants with discrepant PCR result (i.e. positive PCR result in MoH Ref lab and negative PCR result in PHE Porton) had samples analysed in Turkey a median 2 days earlier in disease course than samples analysed in PHE Porton. All participants that were CCHF PCR positive in Turkey were IgM/IgG positive when subsequently tested by ELISA in PHE (results detailed in chapter 5). No participants that were PCR negative in Turkey were found to be PCR positive in PHE Porton. Three participants that were PCR negative in Turkey and PHE, were subsequently found to be IgM/IgG positive by ELISA in PHE and are not included in data analysis. Table 4.1 demonstrates the rates of sample PCR positivity by day of illness. It includes the sample timing of the diagnostic PCR results from Turkey to include the 48 participants that tested positive in Turkey and then negative by PCR in PHE Porton.

Day of illness	Combined (Turkey MoH and PHE) PCR result positive (%)	PHE Porton PCR result positive (569 acute samples)
0	3/3 (100)	n/a
1	11/13 (84.6)	5/7 (71.4)
2	17/25 (68)	8/17 (47.1)
3	31/44 (70.5)	26/37 (70.3)
4	45/71 (63.4)	33/59 (55.9)
5	30/76 (39.5)	27/73 (37.0)
6	22/81 (27.2)	19/79 (24.1)
7	14/76 (18.4)	13/75 (17.3)
8	5/72 (6.9)	5/72 (6.9)
9	5/61 (8.2)	5/61 (8.2)
10	2/44 (4.5)	2/44 (4.5)
11	1/25 (4.0)	1/25 (4.0)
12	0/12 (0.0)	0/12 (0.0)
13	0/8 (0.0)	0/8 (0.0)
14	1/67 (1.5)	1/67 (1.5)
30	0/53 (0.0)	53/53 (0.0)

Table 4.1 Blood samples PCR positive for CCHFV by day of illness in 104 participants.

4.3.1 Viral load at admission

Viral load data at recruitment, shortly after admission was available on the 56/104 participants that tested positive in PHE Porton. The mean viral load at admission was 5.8 log 10 copies/ml (SD 1.17, range 3.2-8.4). The mean viral load was significantly different between the mild severity group (5.52 log 10 copies/ml (SD 1.02, n=35)) and the moderate/severe group (6.30 log 10 copies/ml (SD 1.26, n=21) p=0.022). When patients were stratified by lowest platelet count, the mean log 10 viral load at admission of patients whose platelet count remained greater than 50 x 10^9/L was significantly lower than those that had platelet counts less than 50 x 10^9/L (5.28

(SD 1.01) n=18 vs 6.06 (SD 1.16) n=38, p=0.018). The viral load in fatal patients (n=2, log10 8.26 copies/ml, log 10 7.53 copies/ml) was significantly higher than in patients that survived (p=0.012) (Figure 4.1). There was no significant difference in admission CCHF viral load in patients with bleeding (p=0.61).

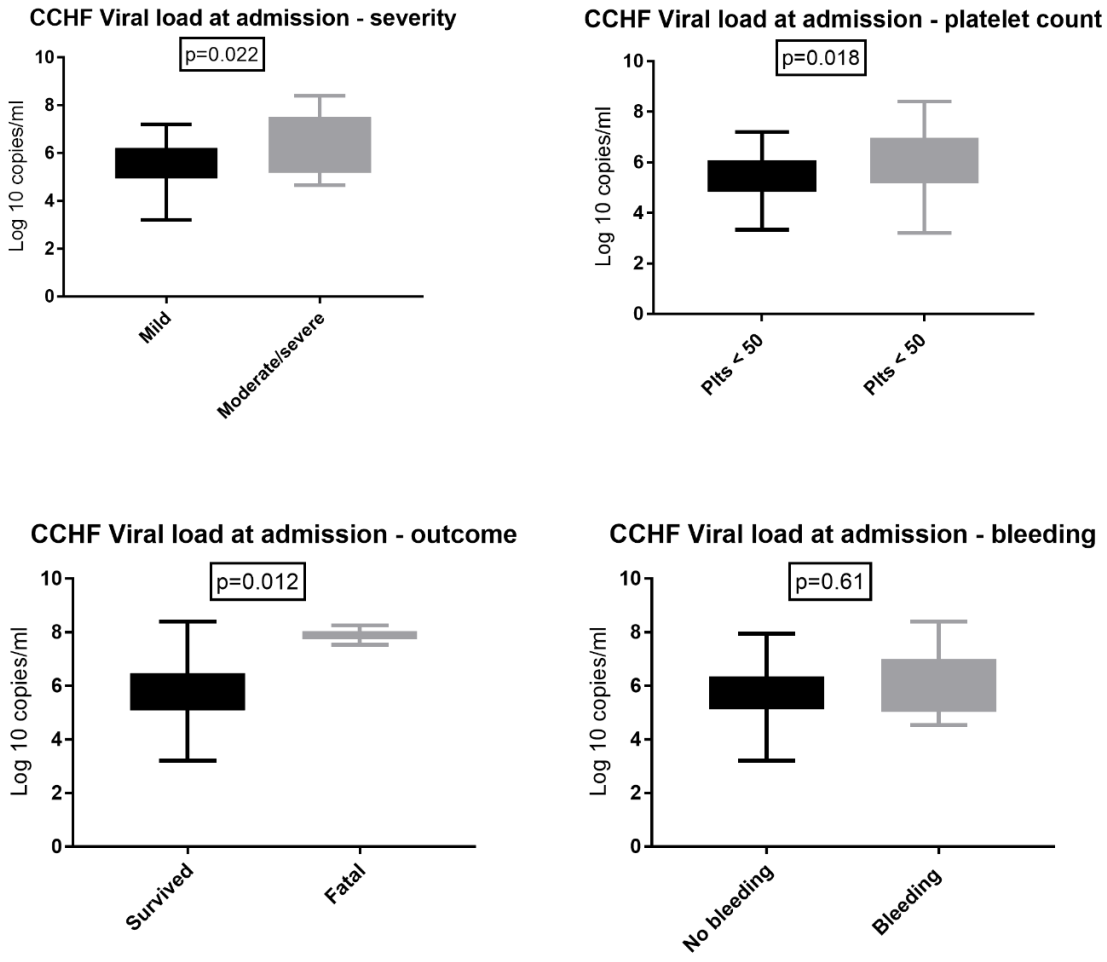


Figure 4.1 CCHF viral load at admission grouped by severity, platelet count, outcome and bleeding**.** Boxplots with whisker represent min to max values. p values are from t tests.

4.3.2 Correlations with admission CCHF viral load

There were no significant correlations between admission viral load (log10 copies/ml) and length of stay (p=0.316) or age (p=0.628). There were no significant correlations shown between admission viral load and admission observation/vital signs (temperature p= 0.240, heart rate p=0.57, respiratory rate p=0.32) but viral load was significantly negatively correlated with systolic blood pressure (p=0.014). No correlation was shown between admission viral load and national early warning score (NEWS p=0.167).

Significant positive correlations with standard laboratory parameters were shown for white blood count (p=0.0003), APTT (p=0.009), PT (P=0.0001), creatinine (p=0.043), LDH (p=0.0002), fibrinogen (p=0.043), and CK (p=0.048). No significant correlations were seen between admission viral load and sodium (p=0.961), potassium (p=0.276), BUN (p=0.708), haemoglobin (p=0.586), haematocrit (p=0.67), AST (p=0.067), ALT (p=0.067) or glucose (p=0.59). Platelet count at admission also did not correlate with viral load at the <0.05 level (p= 0.083), but the platelet count on day 1 and 2 of admission did negatively correlate (p=0.018, p=0.006 respectively).

There were no significant correlations with admission viral load and the CCHF severity scoring index (p=0.11), or with CCHF severity grading system score (p=0.48). There were strong correlations at admission between viral load and the duration of clinical features during hospitalisation (headache p=0.01, myalgia p=0.001, vomiting p=0.008, lethargy p=0.03, fever $>38^0$C p=0.02). A correlation between admission viral load and days of bleeding during hospitalisation was also significant (p=0.001). Participants with a CCHF viral load greater than 6 log 10 copies/ml at admission were significantly more likely to receive blood products during the course of their admission than those <6 log 10 copies/ml (p=0.005) but no differences in whether they developed bleeding during hospitalisation were shown (p=0.772). Participants that that were CCHF PCR positive in PHE porton, were more likely to receive blood products (p=0.002) and have an episode of bleeding (p=0.037).

4.3.3 Serial viral load

Serial CCHFV viral load for all patients is shown on a scatter dot plot with mean and SD in Figure 4.2. Serial mean viral load by day of illness stratified by severity and lowest platelet count is shown in Figure 4.3. Scatter dot plots of serial viral load stratified by severity, lowest platelet and count and the presence of bleeding is also shown in Figure 4.4.

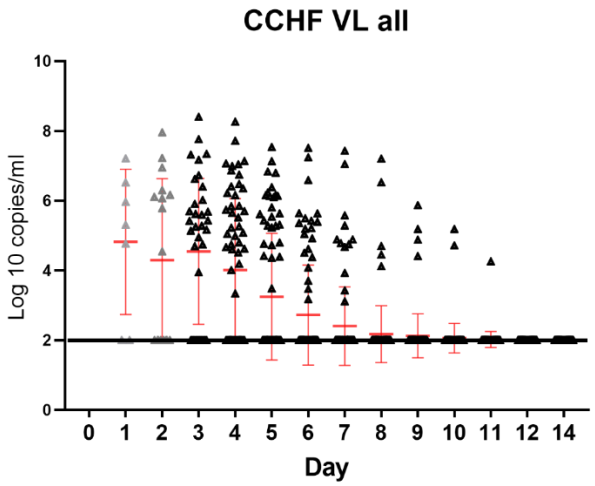


Figure 4.2 Scatter dot plot of CCHFV viral load (Log 10 copies/ml) by day of illness. Mean and SD error bars are shown in red. A line demonstrates 2 log 10 copies/ml L as the limit of detection.

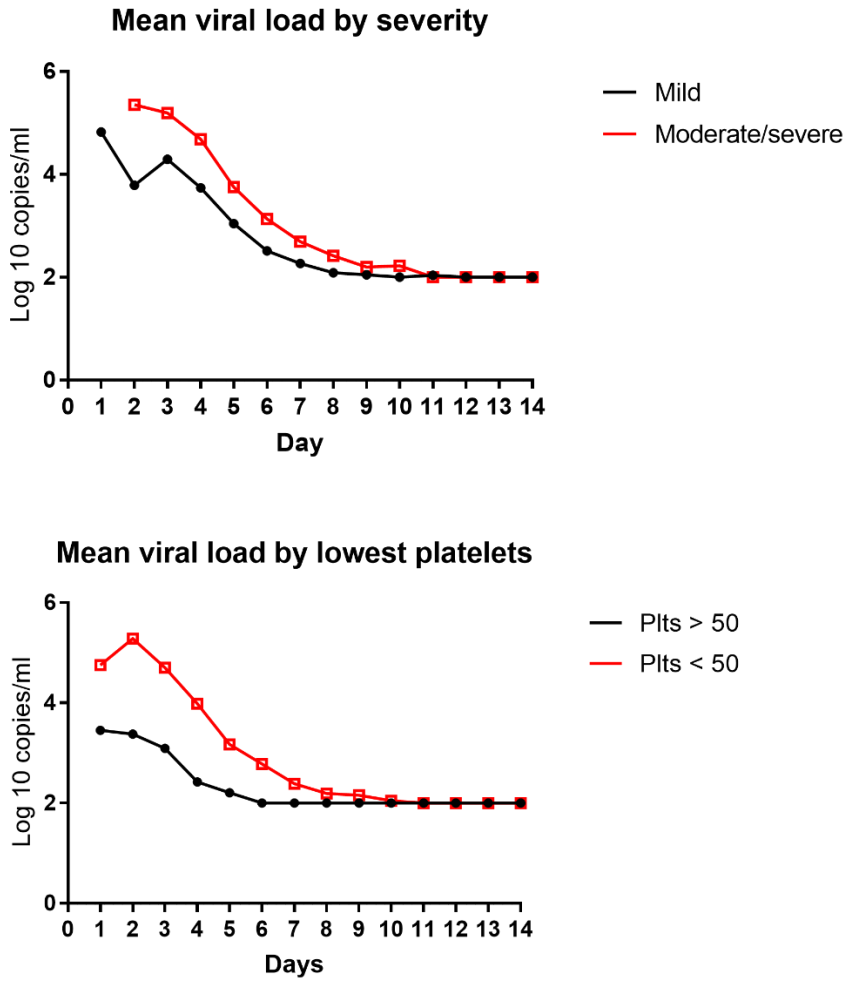


Figure 4.3 Mean CCHF viral load by day of illness stratified by disease severity and lowest platelet count.

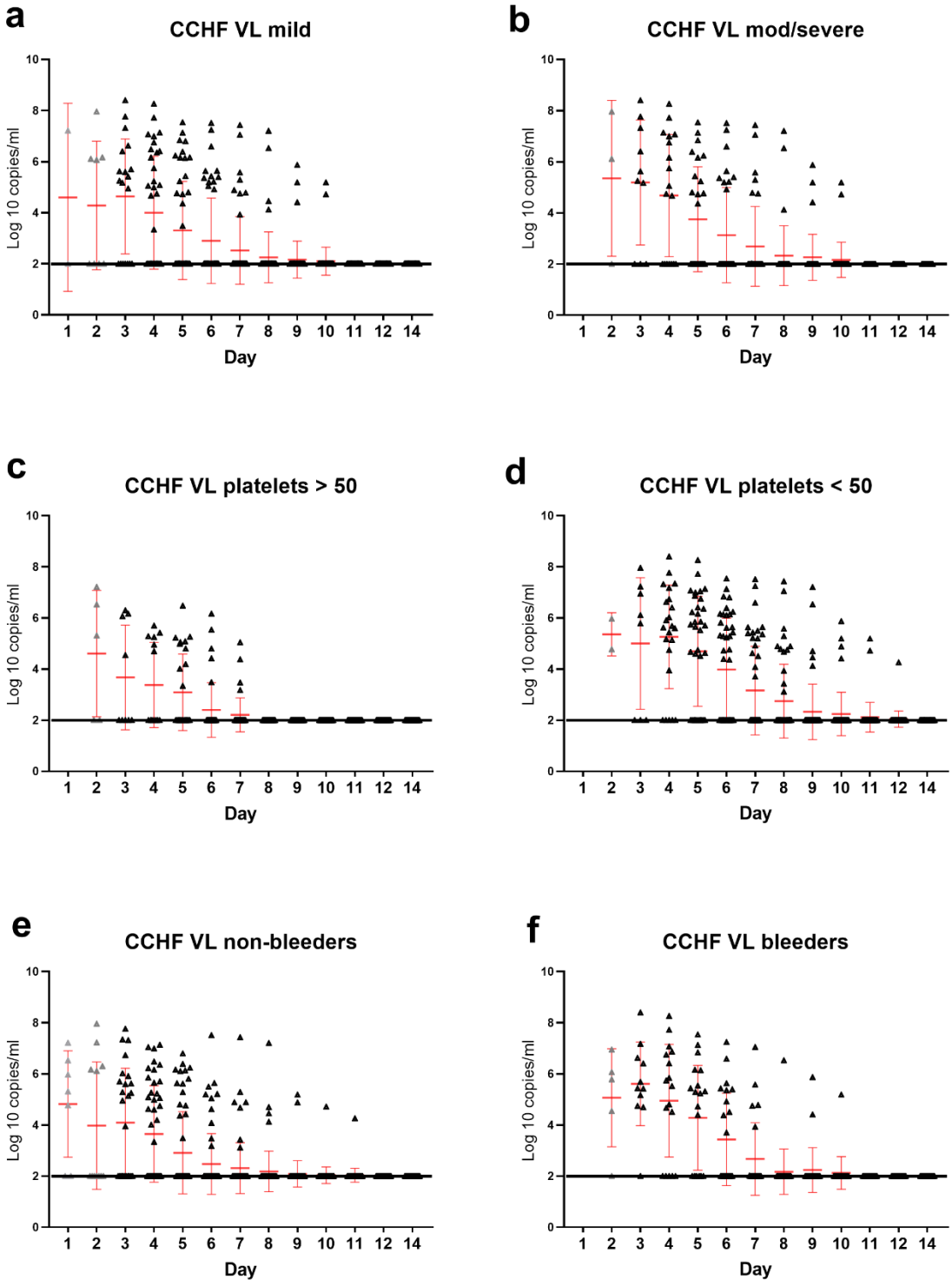


Figure 4.4 Scatter dot plots of serial CCHF viral load by day of illness grouped by severity, lowest platelet count and bleeding. Mean and SD error bars are shown in red. A line demonstrates the 2 log 10 copies/ml limit of detection.

4.3.4 Linear regression analysis viral load

Serial viral load reduction was analysed by linear regression comparing slopes and intercepts/elevations stratified by severity, lowest platelet count and ribavirin use. There was a significant difference between the slopes by severity groups (p=0.0019) and lowest platelet count groups (p<0.0001). No significant difference in slopes (p=0.098) or intercepts/elevation was demonstrated when stratified by ribavirin groups.

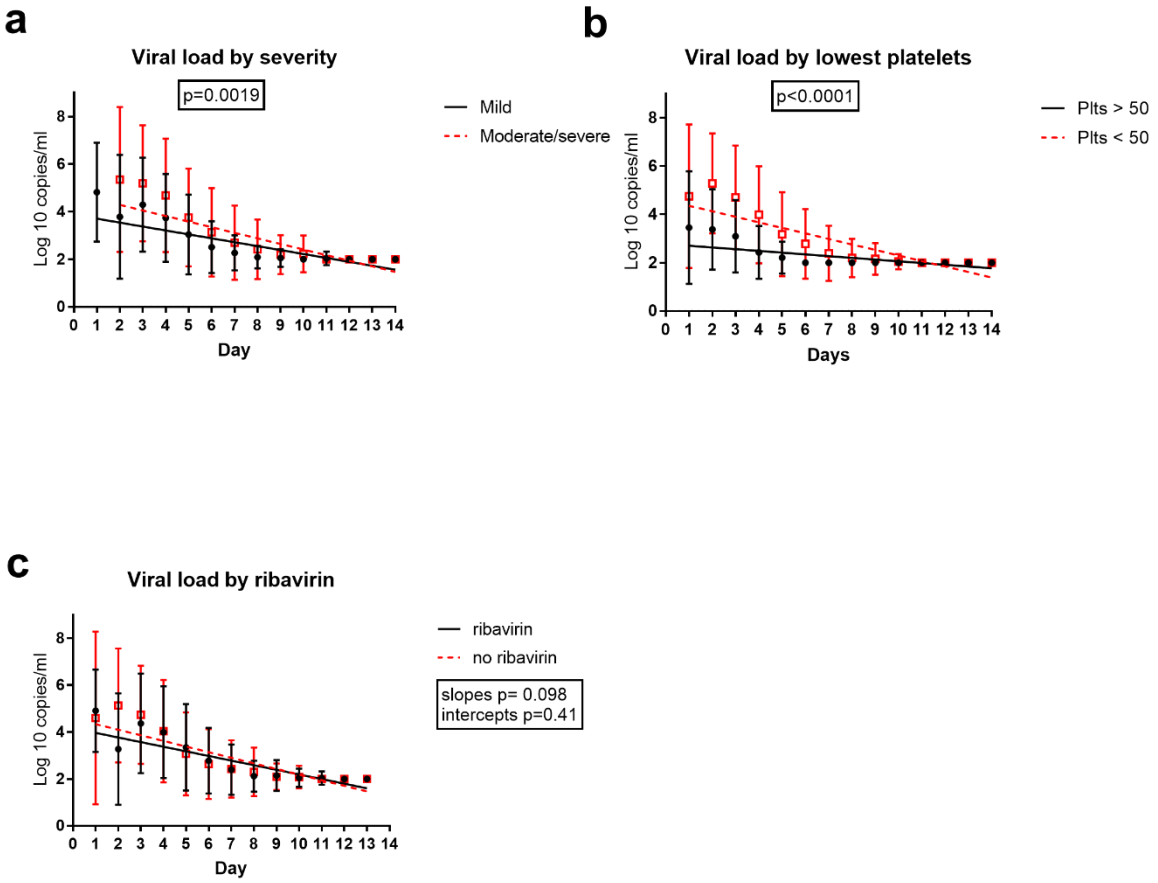


Figure 4.5 Serial CCHF viral load by severity , lowest platelet count and ribavirin use, with group means (SD) and linear regression lines. Slope p-values indicate if regression slopes are equal (a-c). Intercept p-values indicate if the elevations or intercepts are equal.

4.3.5 Viral Load clearance

The clearance of CCHFV from blood (below limit of detection by PCR) by day of illness is shown in Figure 4.6. There was significant difference in rate of clearance of CCHFV by survival curves by severity (p=0.01) and lowest platelet count (p<0.0001). Median clearance of virus occurred at day 5 in mild and day 6 in moderate/severe groups. In participants that developed platelet counts <50 x 10^9/L they also cleared virus later at a median of 6 days, in comparison to day 4 in those maintained platelets >50 x 10^9/L.

In the mild group 38/58 (65.5%) of participants cleared virus by day 5 of illness compared to 14/35 (40%) in the moderate severe group (p=0.02). In the platelet >50 x 10^9/L group 31/41 (75.6%) cleared virus by day 5 compared to 19/52 (36.5%, p<0.0001). Duration of viraemia also positively correlated with a longer length of hospital stay (p=0.03) and number of platelet transfusions received (p=0.002).

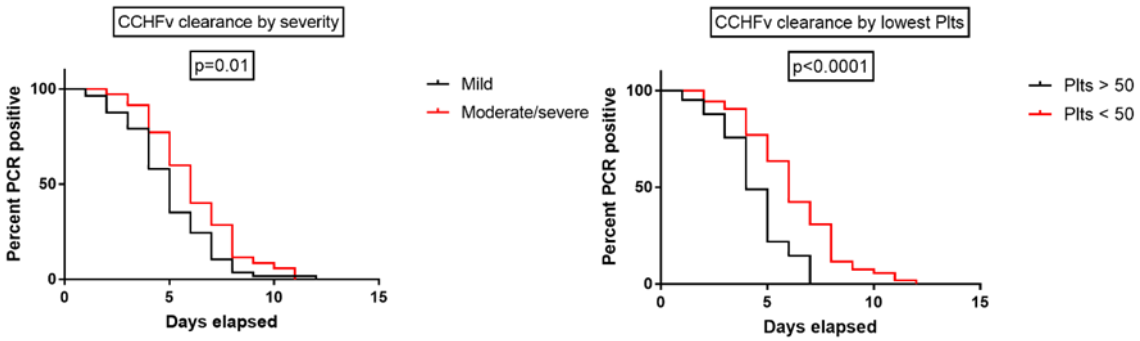


Figure 4.6 Clearance of CCHFV from blood by disease severity and lowest platelets plotted by survival curves. p-values indicate significance of different survival curves (Log-rank (Mantel-Cox) test).

4.3.6 Ribavirin and CCHF VL

Ribavirin was administered to 36/104 participants, all of whom were managed at one study site (Gaziosmanpasa University Hospital) after direct admission or hospital transfer. 16/36 (44.4%) patients that received ribavirin were graded as moderate/severe compared to 25/68 (33.7%) graded moderate/severe in the non-ribavirin group. Viral load at admission was log 5.69 (SD 1.01) in the non-ribavirin group, compared to log 6.23 (SD 1.12) that received ribavirin (p=0.08). At the time that ribavirin was initiated, 25/33 participants were CCHFV positive (3/36 no matching PCR sample on the day of initiated as started prior to recruitment into the study) patients were CCHFV PCR positive, with 8/33 already clearing virus from blood. In total ribavirin therapy was administered for 201 admission days in patients with CCHFV blood PCR results, of which 144/201 were CCHFV PCR negative.

Linear regression analysis showed no significant difference in viral load elimination slopes (p=0.098) between the group that received ribavirin and the group that did not. However, this binary classification of treatment groups risks introducing bias that may limit the potential effect of ribavirin on CHHF VL. This is due to 13/36 participants in the 'ribavirin treatment group' initially being admitted to Tokat State hospital (where ribavirin is not given), and then being transferred to the University Hospital where they received ribavirin. To limit this bias, the data was further analysed comparing the viral load kinetics by day with or without ribavirin administration, using nonlinear mixed effects, shown in Figure 4.7. Patients receiving ribavirin

according to this analysis had a typical elimination rate constant under treatment 56% greater than those that did not receive treatment (p<0.01).

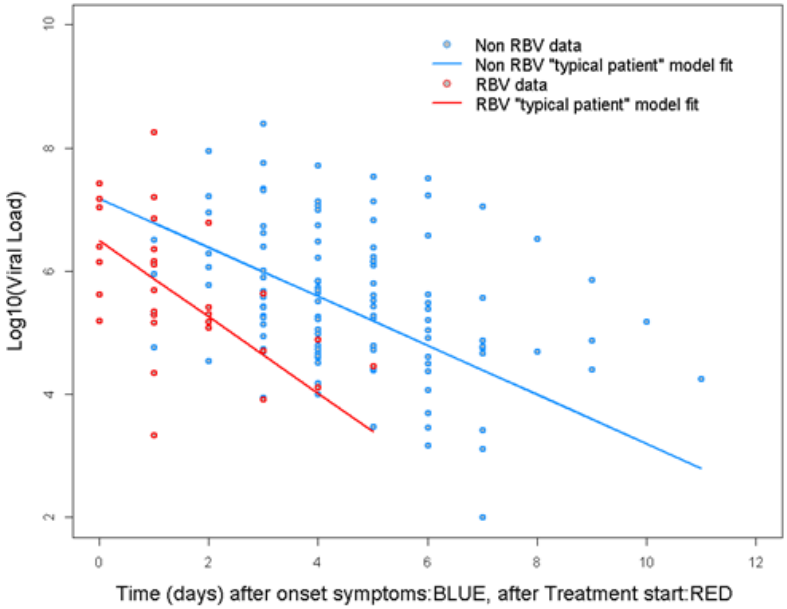


Figure 4.7 Viral load kinetics with or without ribavirin, using non-linear mixed effects model.

4.3.7 CCHFV urine analysis

Urine was collected daily from participants and at 14 and 30 day follow up visits. In total 679 urine samples were processed for CCHF PCR from 104 plasma CCHFV PCR positive patients. This equated to 578 daily samples during the acute admission and 101 from subsequent visits (14 days 54 samples, 30 days 47 samples).

Eight acute CCHF samples were positive by PCR, the majority at Ct values greater than 35 (one sample Ct 30.5, 263,314 copies ml, log 5.5). These samples were all from different participants and were each preceded and followed by negative daily PCR results. Five of the 8 participants with a urine PCR positive result were graded as moderate/severe severity. Plasma was positive at the same time in 2/8 participants with positive urine samples. All convalescent urine samples were negative for CCHFV at 14 days and 30 days.

4.4 Discussion

This is the largest study of CCHFV viral load undertaken to date, with the majority of previous studies evaluating CCHFV viral loads in small patient cohorts at baseline, with Hasanoglu et al [99] in Ankara being the exception that recruited 126 patients for baseline analysis over 6 years. In accordance with this and data published in earlier smaller studies [95–97], we have demonstrated higher CCHF viral loads in fatal cases compared to survivors. We also demonstrated that there is a higher viral load in moderate/severe cases compared to mild cases using a composite severity scoring system. Saksida et al [97] has previously shown this but with different severe and moderate groups. A low platelet count is commonly used in Turkey as an indication to move patients from secondary to tertiary centres. We have shown for the first time that significantly higher CCHF viral loads at admission are associated with the development of platelet counts less than 50 x 10^9/L.

We have demonstrated a correlation between CCHF viral load and bleeding during admission and also shown that viral load correlates with the use of blood products, that was more frequent than bleeding. The data shows a number of correlations between laboratory values and CCHF viral load at admission that include APTT, PT, LDH, CK and creatinine. These frequently form part of severity scoring systems and in practice are used by clinicians to gauge severity of illness and likelihood to require additional supportive care. The correlation with viral load provides further evidence that a simplified scoring system based on viral load could be evaluated. Interestingly there was no direct correlation between viral load and platelet count at the <0.05 level but there was with platelet count in the following days, that suggests a delay in maximal drop of platelets after peak viral load. This may be consistent with direct viral infection of the platelet and subsequent destruction or a delayed immune mediated mechanism resulting in thrombocytopenia. Hasanoglu et al [99] had previously showed a number of significant correlations between admission viral load and variables such as diarrhoea, bleeding, seizure, and requirement for haemodialysis. In this study, as might be expected, the admission viral load correlated with duration of fever. Although this has not been demonstrated previously it concurs with standard practice in the management of EVD and Lassa fever whereby the absence of fever for 48hrs during admission is utilised as a marker for recovery and an indication for PCR testing to allow discharge. Duration of myalgia also correlated with viral load, that is consistent with the CK/viral load correlation and indicates probable viral polymyositis that clinically (and by CK peak levels) was mild, and not at the levels associated with causing renal impairment.

Longitudinal measurements of viral load of viral haemorrhagic fever cohorts have rarely been measured with hypotheses on viral dynamics in blood often crudely inferred from admission sampling at different time points. Only a few small studies have undertaken this in CCHF. The

largest study by Bodur et al [100] investigated VL dynamics in plasma in a case-control study in a cohort of 50 patients, 10 of which received ribavirin. Data is reported by day of admission and relation to ribavirin as opposed to day of illness, but it showed that patients rapidly cleared virus, with no effect of ribavirin demonstrated. This is the first study to show that there is clear differences in CCHF viral dynamics in plasma by disease severity, including the rarely studied 'mild cchf' group and also by those who drop their platelets to less than 50 x 10^9/L, that is most frequently used cut off used in clinical practice to identify those with severe/complicated disease. The main limitation to the interpretation of disease severity viral load slopes is the potential confounding effect of ribavirin, that was more frequently given to the moderate/severe group.

Overall half of patients cleared CCHFV from blood within 5-6 days of onset of symptoms. There were though significant differences in the time of viral clearance by disease severity. Clearance of virus by day 5 occurred in 65% of the mild and 75% of platelet >50 x 10^9/L groups, compared to only 40% in the severe group and 37% in the platelet <50 x 10^9/L groups. This has important infection prevention and control implications and is relevant when considering antiviral treatment and planning for future clinical trials, that may use CCHFV clearance as primary or secondary end-points.

The study was not designed to investigate the potential effect of ribavirin on CCHFV, and when it was initially designed ribavirin was not utilised in the planned study sites. However, after extension to another tertiary specialist centre, one third of the cohort received ribavirin treatment. The use of ribavirin in CCHF has been controversial, with no strong recommendations for use given by WHO, CDC or ECDC. Published meta-analyses are limited by retrospective observational data sets, with the most recent evaluation by the Cochrane reporting that that there was insufficient evidence to show whether ribavirin is effective in treating CCHF, and recommending randomised clinical trials [174]. Even allowing for uncertainty about the effectiveness of ribavirin, the inclusion of patients who received ribavirin has the potential to alter the natural history of disease and viral dynamics. It does however provide an opportunity to observationally evaluate ribavirin treatment, whilst recognising that although not statistically significant, those with moderate/severe disease were more likely to receive it due to the referral mechanisms in place in Tokat.

There was no difference in viral elimination slopes between the group that received ribavirin and the group that did not using linear regression (Figure 4.5). However, this is a binary classification of the two groups, and fails to account for a significant percentage of patients being managed initially without ribavirin, and then it commenced on transfer to a tertiary facility. This has the potential to then underestimate the potential effect of ribavirin on CCHFV

slopes and may explain the difference in Figures 4.5 and 4.7. Figure 4.7 does show a difference between the typical patient model when the ribavirin data is evaluated by day of onset against the non-ribavirin typical patient. This has not been demonstrated before with the best previous study being by Bodur et al [100], who evaluated viral elimination dynamics in a observational study of 10 patients with ribavirin vs 40 control patients. One of the limitations of the ability to demonstrate a benefit of ribavirin therapy in Turkey has been the low case fatality rate which would require unfeasible numbers if powered on a mortality endpoint. Any future RCT is therefore likely to utilise different primary end-points such as viral clearance and elimination slope. This data supports this as a potentially useful strategy.

This is the first practical observation of use of ribavirin in normal clinical practice in Turkey, with matched viral load data. We demonstrated that 25% of patients had cleared virus on the day that ribavirin was commenced, and patients were only PCR positive during 28% of the total number of days that they received ribavirin. This strongly suggests that greater use of CCHFV PCR monitoring, with rapid turnaround of results could improve this and limit the well-recognised adverse sequelae of ribavirin.

There are limited data on the viral dynamics of CCHFV in urine, but a previous report from a small cohort in Kosovo suggested evidence of prolonged viraemia in blood and urine [103]. Urine was shown to be PCR positive in 5 patients that had samples sent to PHE Porton for evaluation. This included a case that was both serum and urine PCR positive at 36 days after onset of symptoms, and another positive on day 6 and 19 when serum was negative. Another case was shown to have variable expression in serum with an unusual pattern (PCR positive day 1,2,18,25 and 34, whilst a negative PCR on sample on day 11). Urine PCR positivity was also variable in the same case with a positive result on day 25, but negative on days 4, 11, 15, 18, 34 and 40.

Due to these results we undertook daily urine sample collection during hospital admission and again at days 14 and 30 when patients attended follow-up. In total 679 urines were processed from the 104 participants, that included 54 samples at day 14 and 47 samples at day 30. All convalescent samples were PCR negative, and only 8/578 acute admission urine samples were PCR positive, all from different participants, with only 2/8 samples PCR positive in blood at the same time. It is clear from these results that urine is rarely CCHFV PCR positive in acute infection and cannot be reliably utilised in convalescence to diagnose recent CCHF infection. The urine and plasma results are noticeably different from the results reported by Thomas et al from Kosovo [103] in 2012, who found prolonged PCR positivity in blood and urine. However, the total number of urines evaluated from confirmed patients is not reported and the samples were not taken as part of a prospective study. The pattern of CCHF clinical disease reported from colleagues in Kosovo is though different than that seen in Turkey (personal communication Salih

Ahmeti). They see a more severe spectrum of disease, with more haemorrhage and higher case fatality rates, and as such may have different viral dynamics. A future prospective study from Kosovo utilising the same RT-PCR, with matched serum results is required to try to re-produce these preliminary results, and to allow further investigation of the different results observed.

In summary this intensive evaluation of CCHF viral load has shown as association with levels by markers of disease severity including platelet count and patient outcome. Viral load has been shown to correlate with clinical features such as bleeding and duration of fever and myalgia, and also with other laboratory variables such as APTT, LDH and CK, with a delayed correlation with platelet count observed. Longitudinal analysis of viral dynamics has again shown difference by CCHF disease severity, with earlier clearance observed in mild cases. For the first time a potential effect of ribavirin on plasma viral elimination slopes has been shown and that urine PCR for CCHFV is not a useful diagnostic test in acute infection or convalescence. Contrary to a previous report the presence of CCHFV in urine is rare occurring in less than 8% of cases and in less than 2% of total admission days.

Chapter 5: CCHFV ELISA

5.1 Introduction

Antibody production in CCHF has been suggested as an important early survival factor, with reduced antibody response demonstrated in fatal cases. The majority of the studies to date suggest that both IgG and IgM antibodies develop early in disease course with IgM becoming detectable from as early as 2-3, but more commonly around 6 days [101]. Small previous studies have suggested that IgG does not become detectable in fatal cases if death occurs before 9 days [97]. No correlation with IgM titre and death or severity has been demonstrated. Previous studies have not undertaken serial daily ELISA but mainly focussed on analysis of diagnostic samples.

5.2 Methods

The detection of immunoglobulins M and G (IgM, IgG) to CCHFV in participant serum samples was undertaken in PHE and utilised a commercial diagnostic enzyme-linked immunosorbent assay (ELISA) kit, VectoCrimean-CHF-IgG/IgM (Vectorbest, Russia).

ELISA is a plate-based assay technique that can be utilised to detect and quantify a range of substances such as antibodies and hormones. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. An indirect ELISA is a two-step ELISA which involves two binding processes of primary antibody and labelled secondary antibody.

1. CL4 laboratory: Samples were thawed and then heat inactivated at 56^{o}C for 30 minutes, with mixing half-way through and then transferred to new tubes in the CL3 laboratory.
2. Preparation of test samples: Test samples were diluted 10 times with the solution for preliminary serum dilution (SPSD), by adding 90ul of SPSD to plate wells and 10Uul of serum with thorough mixing.
3. Washing solution was prepared by diluting the concentrated phosphate buffered saline with Tween (PBS-T) concentrate 25 times with distilled water. Conjugate and Tetramethylbenzidine (TMB) solution is ready to use.
4. Sample addition: 100ul of negative and positive controls, and 10uL of diluted test serum with serum dilution buffer (SDB) was added to all other wells in duplicate.
5. Incubation 1: The plates were sealed with the adhesive film and incubated in the thermostat for 60 minutes at 37^{0}C.
6. Washing 1: Adhesive film was removed and the wells were washed 5 times with the prepared washing solution. 400ul of liquid was added to each well during every cycle with a I minute interval between filling and aspiration
7. Adding conjugate: 100uL was added to each plate well.

8. Incubation 2: Plates were sealed with adhesive film and re-incubated for 90mins at 37^0C.
9. Washing 2: washing repeated as per Step 5.
10. Adding TMB: 100uL of TMB solution was added to each well.
11. Incubation 3: Plate protected from light for 25mins at 18-25^0C.
12. Add stop solution: 100uL of stop solution was added to each well.
13. The optical density (OD) was read on a Molecular Devices SpectraMax M3 absorbance reader and analysed with SoftMax Pro. ELISA background cut-offs were calculated as the mean of plate negative controls (taking highest individual plate value) plus 3 SDs from + 0.2 ($OD_{CO}=OD_{av}C^{-}$ + 3SD + 0.2).

5.3 Results

In total 763 serum samples from 102 participants were analysed for CCHFV IgM/IgG by ELISA. Of these 638 were acute samples taken during hospitalisation, 68 samples were taken at the day 14 follow-up visit and 57 samples at day 30 follow-up. At admission 43/102 (42.2%) patients had a positive CCHFV IgM result, whilst at admission 51/102 (50.0%) had a positive CCHFV IgG. At admission 39/102 (38.2%)participants were IgM negative/IgG negative, 12/102 (11.8%) IgM positive/IgG negative, 20/102 (19.6%) IgM negative/IgG positive, and 31/102 (30.4%) IgM positive/IgG positive (Table 5.1). The proportion and percentage of samples that were positive at each day of acute illness and follow up visits is shown in Table 5.2.

All samples tested after day 8 of clinical illness were IgM positive (149/149), and 96% of IgG samples (143/149) were positive after the same time point. All samples tested positive for IgG after day 11 of illness. All convalescent samples tested at 14 and 30 days after onset of symptoms were IgM and IgG positive. Participants became IgM positive at a median of day 5 (IQR 4-6) of illness (n=100). There was no difference in median day of IgM seroconversion after excluding those that were: IgM positive at admission/first sampling (n=41) and; those without a negative preceding ELISA result 24hrs previously (n=4). Participants became IgG positive at median of day 5 (IQR 4-7) of illness (n=98), with no significant difference compared to the timing of IgM positivity (p=0.923)

IgM titre rose during acute illness reaching a peak at days 9-11 (Figure 5.1). The IgM titre was significantly higher at 14 days in comparison to follow-up at 30 days (p=0.007) . After 8 days of clinical illness the majority of samples tested were positive for IgG (days 9-12 , 143/149, 96%). IgG titre continued to rise during acute infection, with the highest titres recorded on Day 12. Levels continued to rise in convalescence with day 30 having higher levels than day 14 (p<0.0001). Of 39 participants recruited with suspected CCHF that tested PCR negative in Turkey and PHE, 3 participants were found to by IgM/IgG positive (one from each of the study sites),

and 6 participants IgM negative/IgG positive. The 3 participants that were IgM/IgG positive, but PCR negative have not been included in data analysis as after the negative PCR result in Turkey they did not continue with daily sampling.

There was no significant difference in IgM/IgG titres at admission when stratified by CCHF disease severity (IgM p=0.08, IgG p=0.149), although a trend towards higher titres in the moderate/severe group was observed (Figure 5.4). No difference in IgM/IgG titres at admission was noted between fatal or surviving cases at admission. There was also no significant difference in IgM/IgG titres at 5 days (IgM p=0.81, IgG p=0.44), 14 days (IgM p 0.67, IgG p=0.18) or 30 days (IgM p=0.33, IgG p>0.99) when stratified by disease severity (Figure 5.4).

There were 102 patients that had a matched viral load and ELISA results after recruitment, 53 of which had virus detected. In the cohort of patients with detectable VL and ELISA results (n=53) the VL across was significantly different between antibody groups (p=0.01). Viral load was significantly different if the 53 patients were divided into 3 groups (IgM / IgG neg (n=30) median 6.03 Log10 copies/ml, IgM or IgG pos (n= 17) median 5.25 copies/ml and IgM/IgG pos (n=6) median 4.88 log 10 copies/ml, p=0.01) (Figure 5.5). CCHF viral load and both IgM and IgG had strong negative correlations at admission (IgM Pearson r = -0.5571 (95%CI -0.68 to -0.41), p=<0.0001, IgG Pearson r -0.326 (95%CI -0.49to -0.14) p=0.0009). In participants with matched viral load/ELISA data on the day of CCHFV clearance, 40/51 (78.4%) were IgM positive and 37/51 (72.5%) were IgG positive.

	IgM negative	IgM positive	Total
IgG negative	39 (38.2%)	12 (11.8%)	51 (50%)
IgG positive	20 (30.4%)	31 (30.4%)	51 (50%)
Total	59 (57.8%)	43 (42.2%)	102

Table 5.1 Vectorbest CCHFV IgM and IgG ELISA results at admission (n=102)

Day of illness	Proportion of samples IgM positive (%)	Proportion of samples IgG positive (%)
0	0 (0)	0 (0)
1	1/9 (11.1)	2/9 (22.2)
2	5/21 (23.8)	7/20 (35.0)
3	10/37 (27.0)	16/37 (43.2)
4	24/61 (39.3)	23/61 (37.7)
5	48/70 (68.6)	43/70 (61.4)
6	70/78 (89.7)	61/78 (78.2)
7	71/75 (94.7)	64/75 (85.3)
8	65/69 (94.2)	64/69 (92.8)
9	62/62 (100)	58/62 (93.5)
10	45/45 (100)	45/45 (100)
11	27/27 (100)	25/27 (92.6)
12	15/15 (100)	15/15 (100)
14	68/68 (100)	68/68 (100)
30	57/57 (100)	57/57 (100)
365	13/61 (21.3)	(61/61) (100)

Table 5.2 Vectobest CCHFV ELISA IgM and IgG positive rates by day of illness

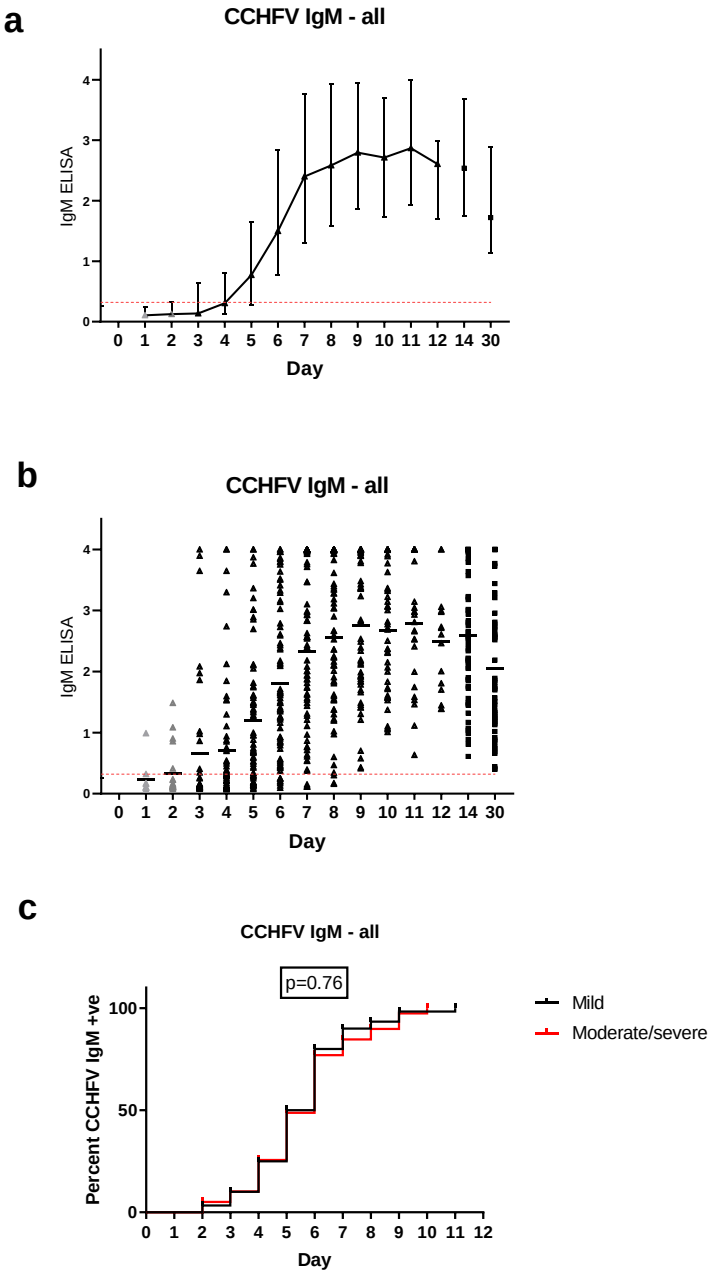


Figure 5.1 Longitudinal IgM ELISA graphs by day of illness. a= Median and IQR . b= Aligned dot plot with mean. y axis units are optical density reads with cut off values displayed. c= Serial IgM ELISA stratified by disease severity (survival curves).

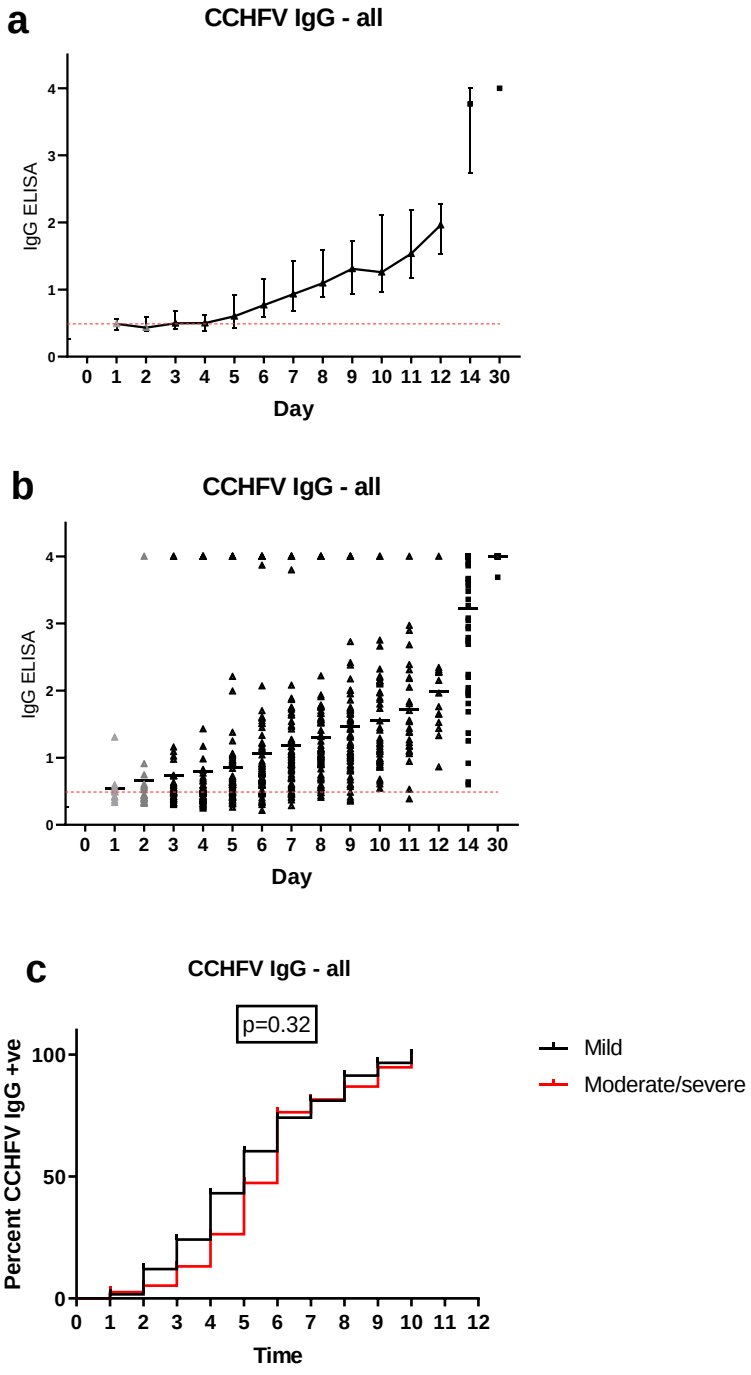


Figure 5.2 Longitudinal IgG ELISA graphs. a= Median and IQR . b= Aligned dot plot with mean. y axis units are optical density reads with cut off values displayed. c= Serial IgG ELISA results stratified by disease severity (survival curves).

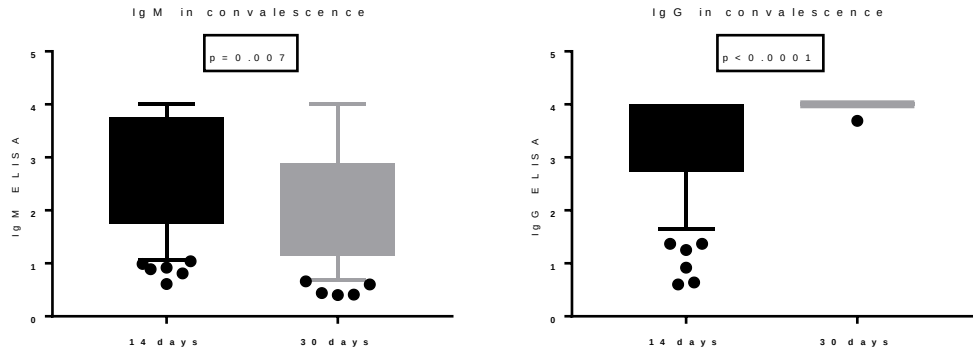


Figure 5.3. IgM and IgG levels in convalescence (IgM/IgG y axis units are optical density reads) Boxplots (whisker represent 10-90th centiles)

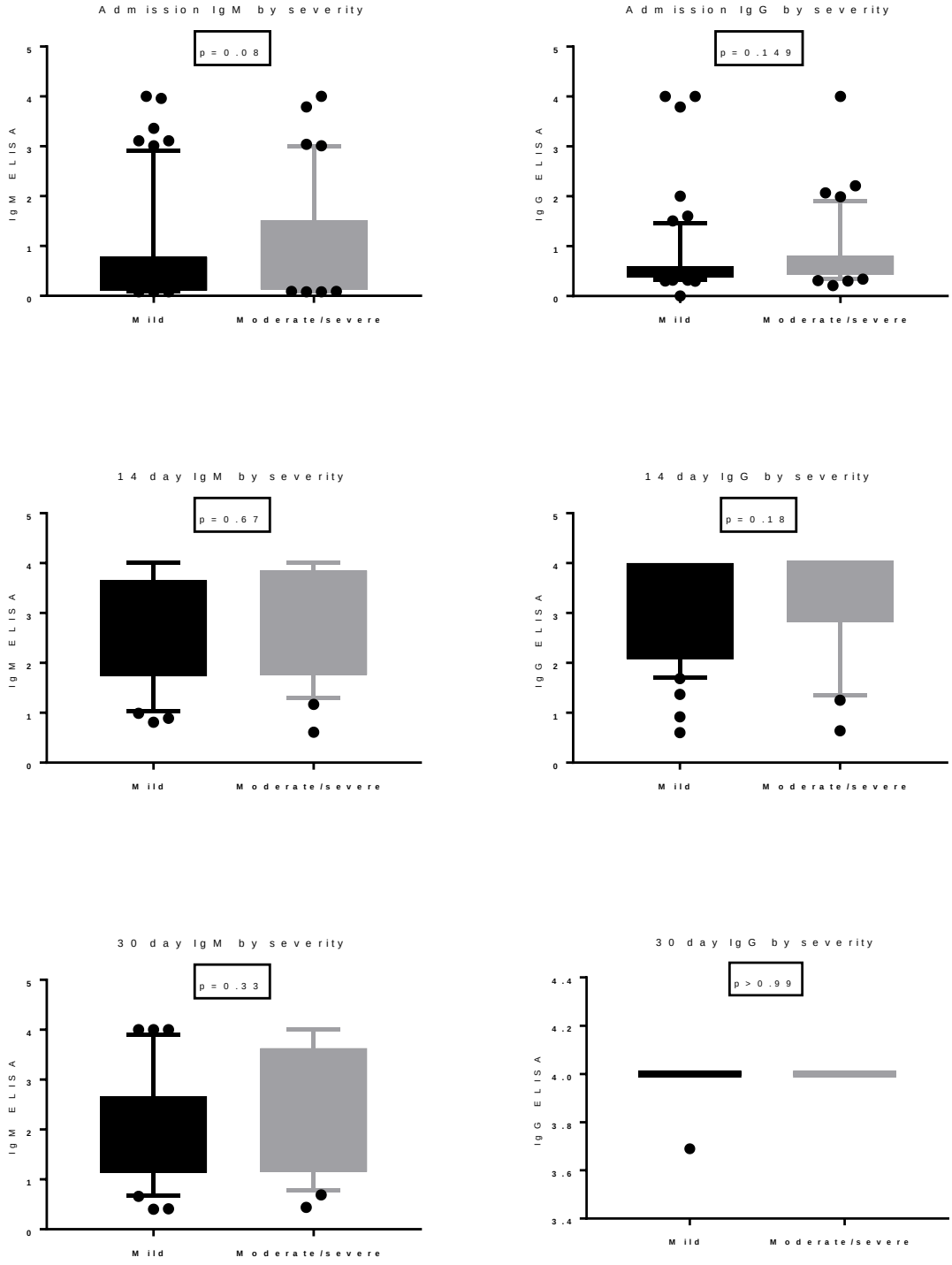


Figure 5.4. CCHFV IgM and IgG ELISA levels at admission, 14 days and 30 days by disease severity. y axis units are optical density reads. Boxplots (whiskers represent 10-90th centiles)

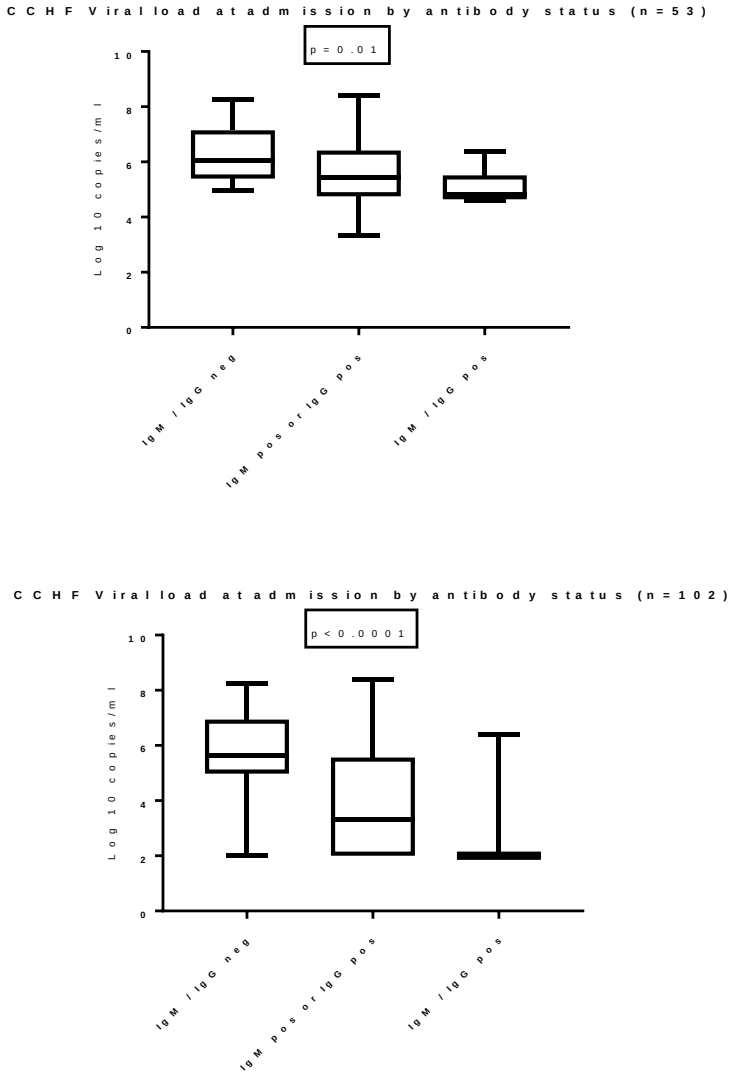


Figure 5.5 CCHF viral load at admission by CCHFV antibody groups. n=53 participants with a matched positive PCR result. n=102 includes participants that were negative by PCR (limit of detection log 2) at study admission sampling. Boxplots (whiskers represent min to max values).

Follow up at 1 year after onset disease

At 1 year follow up (+/-1 month) 61/86 (71%) eligible participants attended or were reviewed in the community with blood sampling. 18/104 were not eligible due to fatal outcome during admission (n=3), location (n=1) being outside the 1 year (+/- 1 month) follow up period (n=14). All participants (61/61) were CCHFV IgG positive at the upper limit of OD, consistent with 30 days results. 13/61 participants were also CCHFV IgM positive at 1 year.

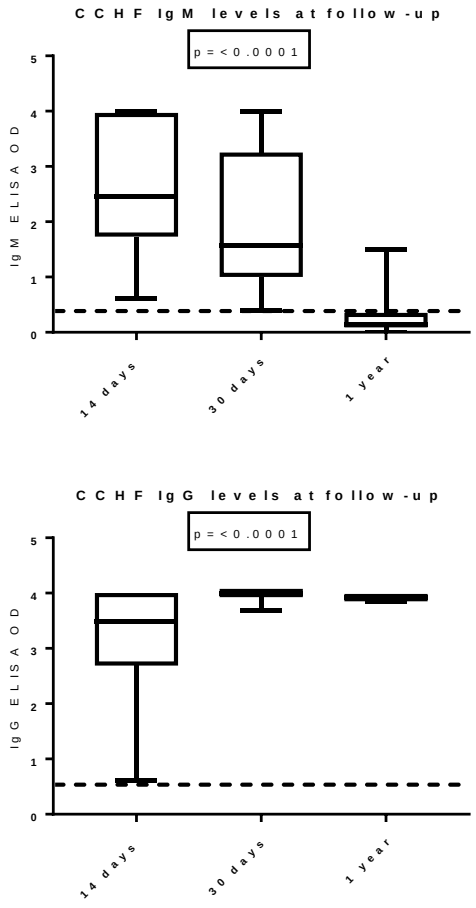


Figure 5.6 CCHFV IgM and IgG ELISA levels at follow-up. Boxplots (whiskers represent min to max values). y axis units are optical density (OD) reads with dotted line as positive result OD cut-off.

5.4 Discussion

The adaptive immune responses to CCHFV have not previously been well characterised. Improved data on the antibody responses to infection is critical for understanding the natural history of infection and has implications for both current diagnosis and the future development of diagnostics and vaccines. CCHF serological responses have previously been evaluated in Turkey during large population seroprevalence studies. In 2009 a large study using the same Vectorbest ELISA kit evaluated 3557 adults, the majority of which were farmers in the CCHF endemic region in Turkey, and showed that 10% overall were positive for CCHF IgG[136]. Our data is the largest and most complete study of IgM and IgG antibody responses to acute infection with CCHFV to date, both in terms of sample size and the total number of samples analysed due to serial daily sampling. It is also the first time that antibody profiles in a cohort of patients with acute CCHF has been followed up at 14, 30 and 365 days.

In total, over 750 samples from 102 participants with PCR confirmed CCHF were analysed for CCHFV IgM/IgG by a commercial ELISA, VectoCrimean-CHF-IgM/IgM produced by Vectorbest. At admission 40.2% of patients had a positive IgM result, with 29.4% of patients being both IgM and IgG positive. Participants became IgM positive after a median 5 days of illness, although as some patients presented with positive IgM results, one would expect the actual median time from that calculated to be shorter. All samples tested after 8 days of clinical illness were shown to be positive for IgM, with levels increasing until days 9-11 (Figure 5.1). IgM remained positive in all convalescent samples tested, although it was significantly lower at 30 days in comparison to 14 days.

At admission 50% of participants had a positive CCHFV IgG result, with the median time for IgG seroconversion again being 5 days. After 8 days of clinical illness the majority of acute samples tested were also positive for IgG (96%). However, unlike IgM titres that began to drop in at the end of acute infection, as might be expected the IgG titre continued to rise until the last sampling point at day 12. Consistent with this and conversely to the IgM pattern, IgG titres were significantly higher at day 30 in comparison to day 14.

These finding are broadly similar to other published data sets, which overall indicate an early and similar IgM/IgG response. Early studies in in South Africa demonstrated CCHF IgG and IgM developing on day 7-9 of illness [26,143]. Later in Kosovo, Saksida et al [97] evaluated the serological response in a cohort of 46 patients with CCHF, of which 15 were fatal cases. Samples were evaluated retrospectively from diagnostic samples a median 6 days (IQR 4-8 days) after onset of symptoms. IgG positivity was only detected in 5/46 patients (all survivors), and IgM in 24/46 of all patients. Further analysis of data showed that the median time of sampling for those with a negative IgM (determined in 15/22) was 5 days (IQR 3-6 days).

More recently Ergunay et al [101] et al, evaluated serological response in Turkey in 20 patients with serial sampling although used a different assay. Although limited clinical data is reported the cohort is similar with only 1/20 fatal cases and a similar duration of viraemia. They found differences with nucleocapsid (N) IgM antibodies becoming positive earlier than glycoprotein precursor IgM antibodies (N-IgM median of 2–3 days after disease onset, GPC-IgM 4–6 days). GPC-IgM and GPC-IgG had a median time to detection of 5 and 6 days respectively. No information is publicly available on the nature of the antigen or CCHFV strain used in the Vector-Best ELISAs, but it is expected to have been validated from strains from south-west Russia.

During our study only participants that were shown to be CCHFV PCR positive in Turkey continued with daily sampling (n=104). Of the participants recruited with suspected CCHF that tested PCR negative in Turkey and PHE, 3/39 participants that were tested by ELISA in PHE were found to be CCHFV IgM/IgG positive, with 6/39 participants CCHFV IgM negative/IgG positive. The aetiology of disease in the 36/39 participants that were CCHFV PCR and IgM negative was not further investigated as part of this study, but in line with previous reports included a range of infective and non-infective causes [206,207].

There was no significant difference in IgM/IgG titres at admission or convalescence when stratified by CCHF disease severity although a trend towards higher titres in the moderate/severe group was observed at admission (Figure 5.4). There was also no difference in IgM/IgG titres at admission between fatal or surviving cases. Saksida et al's [97] cohort included more severe/fatal disease cases, but also demonstrated no relationship between IgM positivity or titre with clinical classification or outcome; only a few IgG positive results generated. Duh et al [96] investigated antibody profiles in 24 patients in Kosovo, including 9 fatal cases and also did not show any correlation with IgM with outcome or CCHF viral load; no patients had detectable IgG in the first 7 days of illness.

Additional previous small studies have also evaluated the relationship between CCHF viral load and antibody response. Wolfel et al [105] demonstrated significant differences in CCHF viral load between those who were IgG positive and negative in 43 samples. Saksida et al [97] also detected a significant inverse correlation of viral load with antibody response with viral load, with RNA levels highest in patients with no detectable antibodies (7.44 log 10 copies per ml) and lowest in patients with detectable IgM and IgG antibodies (4.01 log 10 copies ml – p=0.033). Duh et al also showed that IgG levels after 7 days inversely correlate with VL.

The distribution of viral load across different antibody groups was significantly different. Limiting analysis to only patients with detectable viral loads, participants with the highest viral loads were IgM/IgG negative and those with the lowest in IgM/IgG positive groups (Figure 5.5). This was more significant when all patients with PCR and ELISA results were included (n=102),

with undetectable viral load level inputted at the limit of detection. In accordance with these results strong negative correlations at admission were demonstrated between viral load and both IgM and IgG. There was persistent IgG responses at 1 year that were not significantly different from IgG levels at 30 days, however 20% of participants were IgM positive at 1 year. This is the first long term data on CCHFV antibody responses following acute infection. It shows IgG persistence at 1 year but also that IgM responses may be maintained for long periods in a sub-set of patients. Although IgM has traditionally been considered as a source of transient immunity prior to the development of IgG, recent studies have suggested that it may contribute to natural immunity through specialised subset of B-1 cells [208]. Persistent IgM (> than 1 year) has also been demonstrated in patients that have had Lassa or West Nile virus infection and after yellow fever vaccination [209–211]. Whilst this has important diagnostic implications, the aetiology remains unclear, although it has been hypothesized to occur as a result of strong initial stimulation of the immune system that results in long-term antibody memory [212].

There is limited indirect evidence or knowledge about antibody responses in other viral haemorrhagic fevers to compare with CCHF. In Lassa fever little is known about the immune control of acute infection and recovery has not been shown to correlate with IgG production [213], with neutralizing antibodies only detected after clinical recovery [214]. This is consistent with animal model data such as a study of Lassa fever in cynomolgus macaques by Baize at al [215], that failed to detect neutralising antibodies in up to month after infection in survivors. They did however notice that LV-specific IgM and IgG appeared at earlier stage in survivors.

In EVD data is also limited but IgM antibodies have been shown to appear as early as 2 days after the onset of symptoms, with variable results up to day 9, that then last for up to 30-168 days. IgG antibodies have been shown to develop between day 6-18 and persist for several years [216,217]. Baize et al [218] compared the immune responses of fatal and survivor cases of EVD from two outbreaks in 1996 in Gabon. In survivors, early and increasing titres of IgG, directed mainly against the nucleoprotein and the 40-kDa viral protein, were followed by clearance of circulating viral antigen and activation of cytotoxic T cells. In contrast, fatal infection was characterized by impaired humoral responses, with absent specific IgG and barely detectable IgM.

In dengue, IgM also shown to persist for prolonged period of time [219], that may be useful for serological surveillance as the viremia is short lived. In practice if patients present after 7 days, confirmation relies on serodiagnostic tests. ELISAs are widely available and frequently utilised; however, a single positive dengue IgM result is only considered to indicate a probable case, with seroconversion of IgM or a fourfold increase in IgG antibody titres in paired acute and convalescent serum specimens is required for confirmation. Blacksell et al [220] conducted a meta-

analysis of 11 studies evaluating dengue IgM. The sensitivity and specificity of samples taken up 7 days were shown to be 73% and 88% respectively, compared to later samples (7-10 days) with sensitivity 96% and specificity of 90%.

In summary this data is in accordance with previous data sets reported but has evaluated CCHF antibody responses in much greater depth. It is broadly similar to that reported in EVD with early IgG responses, and persistence of IgM response seen in dengue. The evaluation of the antibody responses in CCHF utilising the Vectorbest kit, is the most detailed and complete undertaken to date and provides important insights into timing and progression of IgM/IgG responses to CCHFV. This will provide important data to the WHO CCHF R&D Blueprint that has identified the qualification of commercial IgM and IgG serological tests using panels of well-characterised clinical samples by 2020 as landmark goal.

Chapter 6 ROTEM Thromboelastography.

6.1 Introduction

Haemorrhage frequently occurs in CCHF and early severity scoring systems [27] reported that platelet counts <20 x 10^9/L, activated partial thromboplastin time (APTT) of greater than 60s or fibrinogen <1.1g/L in the first 5 days of illness were >90% predictive of a fatal outcome [143]. Later scoring systems also incorporated haematological markers and clinical parameters of bleeding [152,154]. In severe disease haemorrhage typically develops after 3-6 days of illness [142] and rates of haemorrhage in Turkey are around 20% [152]. Petechiae, ecchymoses, epistaxis and bleeding from the gastrointestinal, genitourinary and respiratory tracts are the most common haemorrhagic manifestations [180] and have been shown to be associated with a poor clinical outcome [147,148].

The evolution and pathogenesis of coagulopathy of viral haemorrhagic fevers are poorly understood and there are few data about the coagulopathy of CCHF. Protein S and protein C levels, activated protein C resistance and D-dimer levels were not associated with mortality in one study of 83 patients with CCHF [190]. Thrombocytopenia and platelet dysfunction have been demonstrated in Lassa fever [168,184,221] and in Ebola virus disease [204].

Traditional screening coagulation tests such as APTT were first developed whilst discovering the coagulation cascade [222], and provide information about coagulation factor deficiencies, but not about the full haemostatic capacity of clot formation, thrombin generation, platelet number and function, or fibrinolytic activation. They also have a laboratory turnaround time that can delay therapeutic interventions. Rotational thromboelastometry (ROTEM) is a near-patient assay giving a global haemostatic picture, in that it can give information on all haemostatic parameters. It is derived from a technique that was first developed in the 1940's, and analyses three phases of coagulation (initiation, amplification, and propagation), reflecting the interactions of the cellular and plasma components of coagulation and the activity of the fibrinolytic system [223]. A whole blood sample is placed in a cuvette and a cylindrical pin immersed, maintaining a gap bridged by the blood. The pin is rotated and, as the blood begins to clot, its movement is restricted and the changes in mechanical kinetics that result can be plotted to generate typical curves (TEMogram) and numerical parameters (Figure 6.1).

ROTEM values have been used to detect and manage coagulopathy in haemorrhagic disorders including trauma, post-partum haemorrhage, intensive care, and surgery including liver transplantation. The National Institute of Clinical Excellence (NICE) in the UK has recommended its use during cardiac surgery as it has been associated with improved clinical outcomes [222,224,225]. ROTEM analysis of a cohort of 53 patients with dengue demonstrated significant impairment in thromboelastometry values [226]. A similar technique, thromboelastography (TEG)

was undertaken on two cases of Ebola virus disease in the UK [227], showing evidence of a coagulopathy, followed by hypercoagulability on recovery.

Aim

The aim of this study was to undertake ROTEM analysis alongside conventional coagulation testing in a cohort of patients with CCHF to increase understanding of the coagulopathy.

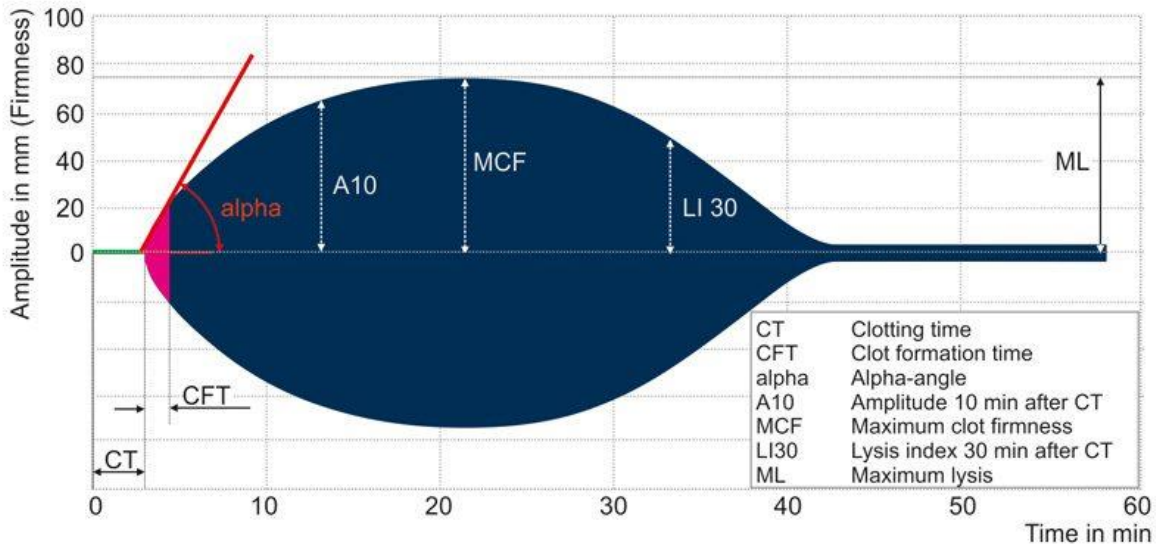


Figure 6.1 ROTEM® thromboelastometry parameters and scaling (from www.rotem.de)

6.2 Methods

Study Design

Prospective observational cohort study. Demographic, clinical and laboratory data were collected from patients consecutively admitted with confirmed CCHF in hospitals in Samsun and Tokat, Turkey, between May and August 2015. Blood samples for ROTEM analysis were drawn during the course of hospital admission, and in convalescence (day 14-30). As previously described all participants had CCHF severity assessment undertaken by Swanepoel criteria [143], severity grading score (SGS) [152] and severity scoring index (SSI) scoring systems [154](Table 1.6). Patients were given blood products according to clinician assessment and recorded daily.

Blood sampling

Routine haematology variables were analysed using automated analysers in local ISO / IEC 17025 and ISO 15189 standard accredited laboratories. The normal ranges for Prothrombin time (PT) and APTT were 11-15 and 27-45 seconds respectively. Standard ROTEM analysis was performed on 3.2% citrated whole blood using a ROTEM delta point of care analyser (Tem International, Germany) according to the manufacturer's instructions, less than 2 hours after

blood draw. Single use EXTEM S and FIBTEM S assay reagents were used with an automated pipette for standardised volumes. Analysis was performed inside a Class 2a BSC, with the operator wearing personal protective equipment. EXTEM S screening was used which gives information on clot formation particularly through the extrinsic pathway and FIBTEM S which isolates coagulation and provides information on the contribution of fibrinogen to clot firmness. The following EXTEM (S) variables were recorded (normal range): Clotting time (CT, time from start of the measurement until initiation of clotting - 38-79 seconds); Clot formation time (CFT, time from initiation of clotting until a clot firmness of 20 mm is detected- 34-159 seconds); Amplitude at 10 minutes after CT (A10, clot firmness 10 mins after clot initiation - 43-65mm); Maximum clot firmness (MCF, clot firmness/ overall clot stability - 50-72mm); Maximum lysis (ML, >15% at 1 hour). The following FIBTEM S variables were recorded: A10 (7-23mm); and MCF (9-25mm).

Statistical Analysis

Descriptive analyses are reported as frequencies (proportions) for categorical variables and means (standard deviation or 95% confidence interval (CI)) or medians (ranges) as appropriate for continuous variables. Categorical variables were compared between sub-groups with the Fisher exact test. Continuous variables measured at admission were compared using Student t-tests or the Mann-Whitney U-test/Kruskal-Wallis test as appropriate. Continuous variables measured longitudinally were compared using linear regression models with robust standard errors and adjustment for clustering of measures within patients. No imputation for missing data was made due to small sample sizes. Hypothesis tests were two-tailed ($p<0{\cdot}05$) and analyses were performed using the SPSS (version 24) and Stata (version 14) computer packages.

6.3 Results

6.3.1 Demographics and sampling

During the study period 65 patients with confirmed CCHF were recruited, with 49/65 (75%) graded mild illness severity and 2/65 (3.1%, 95%CI 0.4% - 10.7%) cases having a fatal outcome. Demographic information is detailed in Table 6.1. In total, 212 ROTEM analyses (EXTEM S n= 107, FIBTEM S n= 105) were performed. In 45/65 participants, baseline ROTEM analysis was undertaken within 48 hours of admission, of whom 31/45 (69%) were graded mild illness severity. Of the remaining 20/65, 14/20 had ROTEM analysis after this time point (48hrs) during acute admission and 6/20 only at follow-up during convalescence. In total 110 blood samples were analysed and grouped by day of illness: day 0-3 (n=27); day 4-6 (n=46): day 7-10 (n= 25); and convalescence (n=12, median 21 days after onset of illness, IQR 17-30 days).

6.3.2 Clinical features and case management

Haemorrhage occurred in 13/65 (20%, 95%CI 11.1-31.8%) participants, who had a total of 25 days of haemorrhage during 424 days of study observation. The site of bleeding was: oral (7/13); nasal (7/13); sputum (3/13); vomit (2/13); stool (2/13); intravenous puncture sites (2/13); vaginal (1/13); urine (1/13) and skin (1/13). Thirty-five participants developed platelet counts <50 x 10^9/L during admission (35/65, 54%), with 11/65 (17%) having a platelet count <20 x 10^9/L.

Fresh frozen plasma was administered to 15/65 (23%) participants, red cell concentrates to 8/65 (12%) and platelet transfusion to 21/65 (32%) participants. Eleven of 13 (84.6%) patients who developed haemorrhage had received blood component therapy prior to the onset of bleeding. At the time of bleeding (25 patient admission days), median platelet count was 44 x 10^9/L (range 4-152 x 10^9/L), median PT 13 seconds (range 11-38 s, 23/24 within normal limits) and median APTT was 48 seconds (range 22-85 s, 10/24 within normal limits). At the time of bleeding vital signs were within normal limits for 17/25 participants, with only one having a qSOFA score ≥2 and national early warning score (NEWS) >3 (0-4 no action). NEWS score of >3 was utilised as oxygen saturations were not routinely available. Ribavirin treatment was given to 35/65 (54%) participants at standard dosing.

6.3.3 ROTEM at admission

Haematological parameters on admission (n=45) are shown in Table 6.2. Those with moderate/severe disease had significantly lower platelet counts ($p<0.001$) and more prolonged APTT ($p<0.001$). ROTEM analysis at admission demonstrated significant differences in all EXTEM S parameters by disease severity, with CT, A10, CFT and MCF all having more abnormal values in the moderate/severe group when compared to the mild group (Table 6.2, Figure 6.2). There was no evidence of hyperfibrinolysis (EXTEM S LI60 >15%) at 60 minutes (0/44). There were significant correlations between maximal clot firmness, clot formation time and A10 with platelet count ($p<0.0001$). PT and APTT both correlated with clotting time ($p<0.0001$) (Figure 6.4). Importantly for a disease thought to be associated with disseminated intravascular coagulation, there were no significant differences in FIBTEM S A10 or MCF between the disease severity groups.

	All (n=65)	Mild (n=49)	Moderate/ severe (n=16)	p-value
Gender (%):				
- Male	40 (61.5)	31 (63.3)	9 (56.3)	
- Female	25 (38.5)	18 (36.7)	7 (43.7)	0.77
Age (years)				
- Mean (SD)	53 (15.6)	53.9 (16.6)	50.3 (11.8)	0.43
Tick bite (%):				
- Yes	45 (69.2)	36 (73.5)	9 (56.3)	
- No	20 (30.8)	13 (26.5)	7 (43.7)	0.22
Mode of admission (%):				
- Direct	56 (86.2)	44 (90.0)	12 (75.0)	
- Hospital transfer	9 (13.8)	5 (10.0)	4 (25.0)	0.21
Time from symptom onset to admission (days) - Median (range)	2 (0-7)	3 (1-8)	4 (1-8)	0.07
Length of admission (days)				
- Median (range)	8 (2-16)	8 (3-16)	8 (2-11)	0.45
Fatal outcome (%):				
- Yes	2 (3.1)	0 (0)	2 (12.5)	
- No	63 (96.9)	49 (100)	14 (87.5)	0.06
Haemorrhage during admission (%):				
- Yes	13 (20.0)	7 (14.3)	6 (37.5)	
- No	52 (7.0)	42 (85.7)	10 (62.5)	0.07
Blood product replacement (%):				
- Platelets	21 (32.3)	13 (26.5)	9 (56.3)	0.03
- Fresh frozen plasma	15 (23.1)	7 (14.3)	9 (56.3)	0.002
- Red Blood cells	8 (12.3)	3 (6.1)	5 (31.3)	0.02
Ribavirin treatment (%):				
- Yes	35 (53.8)	23 (47.0)	12 (75.0)	
- No	30 (46.2)	26 (53.0)	4 (25.0)	0.08

Table 6.1 Demographics, clinical features and treatment characteristics of CCHF cases with ROTEM analysis

ROTEM parameter	All (n=45)	Mild (n=31)	Moderate/severe (n=14)	P-value
Platelets Median (range)	73 (4-162)	90 (46-166)	46 (4-108)	<0.001
WBC Median (range)	2400 (740-28300)	2350(740-5310)	2450 (850-28300)	0.741
Prothrombin time (s) Mean (SD) *	14.0 (4.4)	13.1 (2.1)	15.8 (7.0)	0.076
APTT Mean (SD) *	38.3 (13.8)	32.8 (6.7)	49.9 (17.8)	<0.001
Haemoglobin Mean (SD)	13.5 (2.1)	13.8 (1.8)	12.7 (2.6)	0.103
EXTEM S CT (s) Median (range) 38-79 (number) >79 (number)	 59 (42-164) 42 3	 56 (42-81) 30 1	 68.5 (48-164) 12 2	 0.01
EXTEM S A10 (mm) Mean (SD) 43-65 <43	 41.6 (9.1) 19 26	 45.1 (7.0) 17 14	 33.9 (8.6) 2 12	 <0.001
EXTEM S CFT (s) Median (range) 34 - 159 (normal range) 160 - 220 (usually unimpaired haemostasis with reduced reserve) 221 - 300 (bleeding risk) 300 - 400 (high bleeding risk) >400 (usually no effective haemostasis)	 165 (72-418) 20 17 6 0 2	 147 (72-255) 16 13 2 0 0	 197 (98-418) 4 4 4 0 2	 0.006
EXTEM S MCF (mm) Mean (SD) >50 (normal range) 46-49 (usually unimpaired haemostasis with reduced reserve) 40-45 (bleeding risk) 30-40 (high bleeding risk) <30 (usually no effective haemostasis)	 51.5 (10.1) 24 12 6 2 1	 54.4 (7.2) 21 7 3 0 0	 45.1 (12.5) 3 5 3 2 1	 0.003
EXTEM S LI60 >15%	 0	 0	 0	 N/A
FIBTEM S A10 (mm) Median (range) 7-23 <7	 13 (6-25) 42 1	 13 (8-20) 29 0	 11.5 (6-25) 13 1	 0.677
FIBTEM S MCF (mm) Median (range) 9-25 <9	 15 (7-60) 41 2	 15 (9-60) 29 0	 16.5 (7-39) 12 2	 0.209

Table 6.2 Haematology and ROTEM data at admission(<48hrs) by severity (total n=45). * indicates 28 patients in mild and 13 patients in moderate/severe groups with admission PT/APTT results

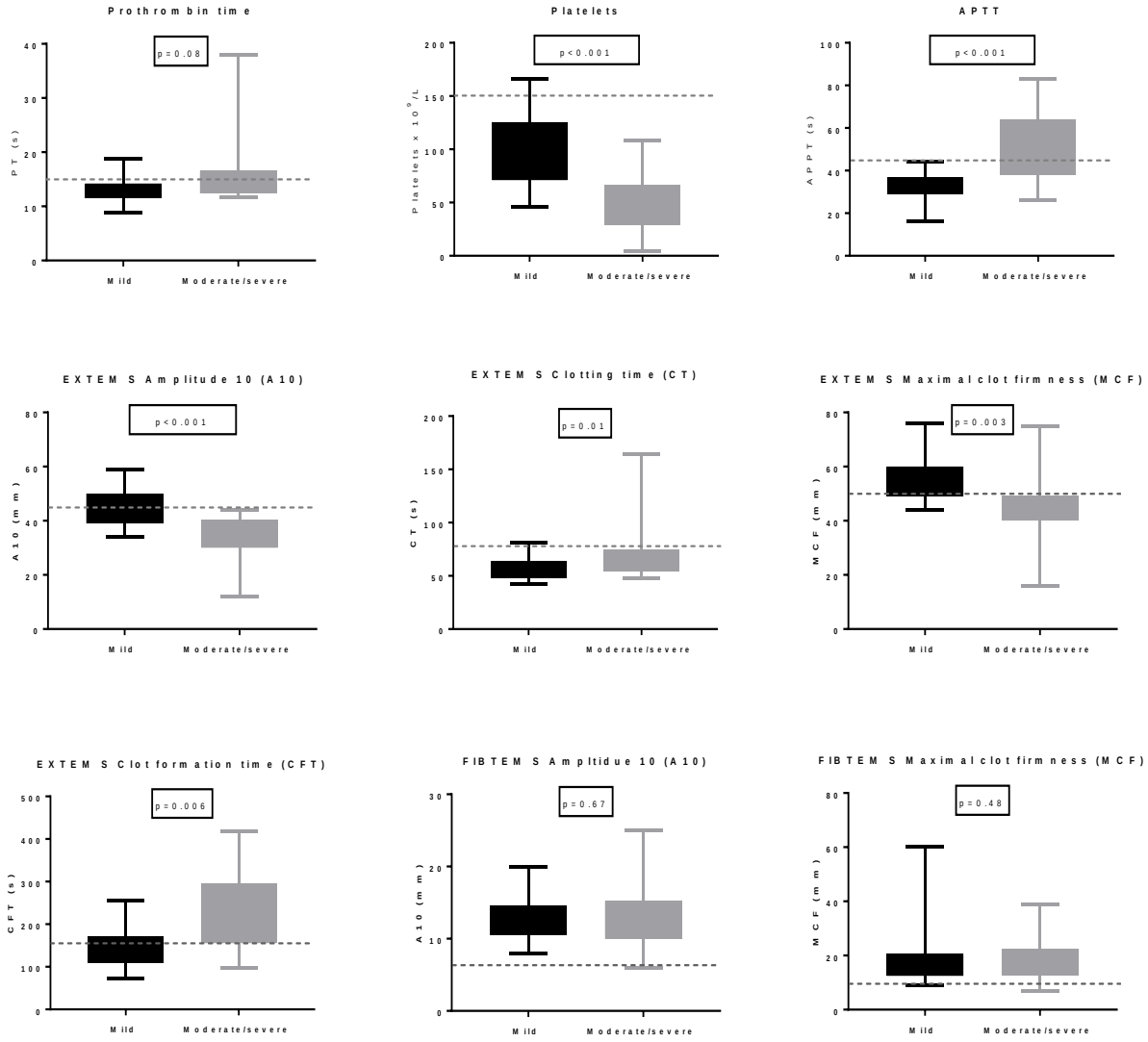


Figure 6.2 Box and whisker plots of haematology, coagulation and ROTEM findings at admission (n=45) stratified by CCHF disease severity. Boxes represent median, 25th and 75th centiles. Whiskers represent maximum and minimum values. Dotted lines represent upper (PT, APTT, CT, CFT) and lower limits (platelets, A10, MCF) of normal.

Among the patients with EXTEM S CFT measurements that demonstrated increased bleeding risk (CFT >221s), 16/25 (64%) had platelet counts >20 x 10^9/L and 5/25 (20%) had counts >50 x 10^9/L. Of the patients with EXTEM S MCF measurements demonstrating bleeding risk (MCF < 40mm), 15/24 (62.5%) had platelet counts >20 x 10^9/L and 3/25 (12%) had counts >50 x 10^9/L, again suggesting that the platelet count was not the determining factor of bleeding risk.

6.3.4 ROTEM changes according to day of illness

There was no significant difference in CT by day of illness (p=0.68). EXTEM S A10 and MCF analysis was different by day of illness, with lowest values occurring on days 4-6 (p=0.090 and p=0.024 respectively). There was no significant difference in EXTEM S LI60 by day of illness, with no abnormal results. FIBTEM S A10 showed significant differences by day of illness in acute samples with Days 0-3 and 4-6 having lowest values (p=0.003), but only 1/93 FIBTEM values was lower than normal (<7s). FIBTEM S MCF was also lowest on days 0-3 and 4-6 (p=0.082). All EXTEM S and FIBTEM S parameters were within normal ranges in convalescence (n=12). There were significant differences between EXTEM S acute and convalescent CT (p=0.013), A10 (p<0.001), CFT (p<0.001) and MCF (p<0.001) values but no significant differences in FIBTEM S between acute and convalescent samples (Table 6.3).

A typical case of CCHF with ROTEM data, standard haematology/coagulation parameters and TEMOGRAMs is displayed in Figure 6.3. Two fatal cases of CCHF were included in the study. The first case was a 69-year old male who had no episodes of bleeding, normal ROTEM parameters and died due to a hospital acquired infection. The second case was a 52-year old female who presented on day 4 of illness and then rapidly developed septic shock with minor bleeding from the oral mucosa, nose and intravenous access sites. Bloods results at admission were: aPTT =120 secs; PT= 40 secs; platelets = 28 x 10^9/L. ROTEM values at admission were grossly abnormal: EXTEM S CT 164 secs; A10 = 33 mm; CFT=240 secs; MCF 46mm; LI60 <15% (no evidence of hyperfibrinolysis); FIBTEM A10 = 7mm; and MCF = 7mm. She progressed to multi-organ failure, dying within 36 hours of admission, despite ribavirin treatment. She received 2 units of platelets and 6 units of FFP on each days of admission

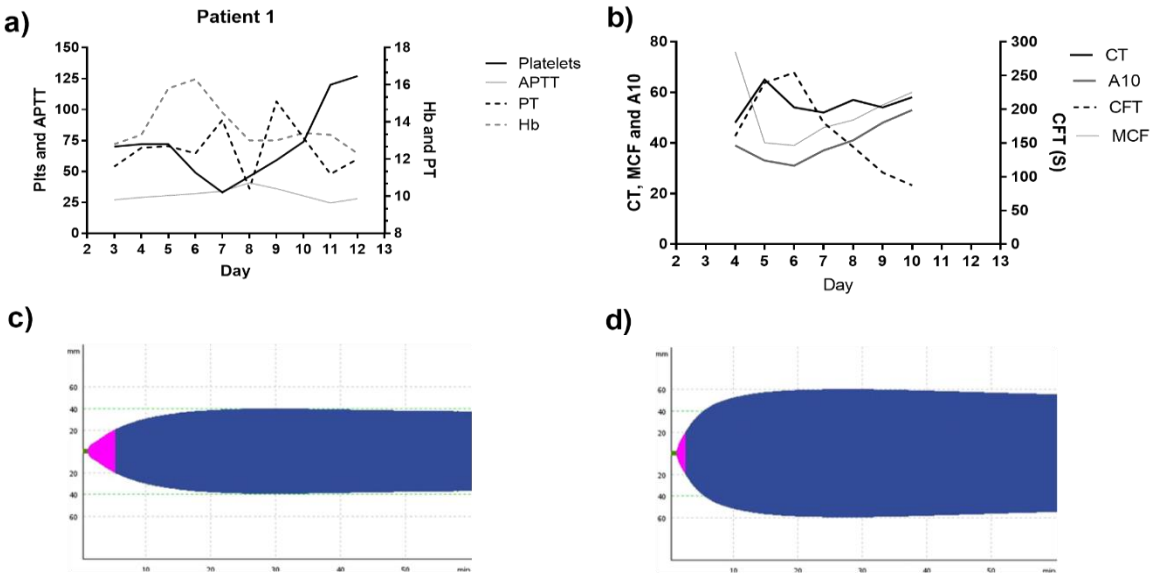


Figure 6.3 Illustrative case with serial ROTEM analysis: Case history: 30-year old male admitted 3 days after a tick bite with a 1-day history of fever, lethargy, headache and anorexia. He developed gingival bleeding on day 7 and 8 of illness and was discharged on day 11 of illness. He did not receive any blood products during admission. a) Haematology and coagulation parameters. b) ROTEM EXTEM S parameters by day of illness c) EXTEM S TEMOGRAM at day 6 of illness. d) ROTEM EXTEM S TEMOGRAM at day 10 of illness.

ROTEM parameter		Acute Day 1-3	Acute Day 4-6	Acute Day 7-10	p-value	Acute all	Convalescent Day 14-30	p-value
EXTEM S CT (n=107)	Mean (95% CI)	61.0 (55.8 : 66.2)	64.1 (56.5 : 71.6)	59.7 (52.1 : 67.3)	0.686	62.1 (57.6 : 66.6)	50.7 (47.0 : 54.3)	<0.001
38-79	n	24	38	21		83	13	
>79	n	2	6	3		11	0	
EXTEM S A10 (n=107)	Mean (95% CI)	42.5 (39.0 : 46.1)	37.9 (34.6 : 41.2)	42.6 (38.3 : 46.9)	0.090	40.4 (38.3 : 42.5)	62.9 (58.3 – 67.5)	<0.001
43-65	n	11	15	13		39	13	
<43	n	15	29	11		55	0	
EXTEM S CFT (n=107)	Mean (95% CI)	168 (137 : 200)	210 (169 : 250)	155 (124 : 187)	0.097	184 (161 : 207)	71 (55 : 88)	<0.001
34 - 159 (normal range)	n	10	18	15		43	13	
160 - 220 (usually unimpaired haemostasis with reduced reserve)	n	12	10	5		27	0	
221 - 300 (bleeding risk)	n	3	10	3		16	0	
300 - 400 (high bleeding risk)	n	0	3	1		4	0	
>400 (usually no effective haemostasis)	n	1	3	0		4	0	
EXTEM S MCF (n=102)	Mean (95% CI)	52.3 (48.7 : 55.9)	48.7 (45.2 : 52.2)	55.5 (52.2 : 58.8)	0.024	51.5 (49.4 : 53.5)	68.5 (64.8 : 72.3)	<0.001
>50 (normal range)	n	13	22	16		47	13	
46-49 (usually unimpaired haemostasis with reduced reserve)	n	9	5	2		16	0	
40-45 (bleeding risk)	n	3	9	4		16	0	
30-40 (high bleeding risk)	n	1	4	1		6	0	
<30 (usually no effective haemostasis)	n	0	2	0		4	0	
EXTEM S LI60 (n=93)	Mean (95% CI)	95.0 (93.6 : 96.4)	97.9 (96.9 : 98.8)	96.4 (94.4 : 98.4)	<0.001	96.7 (95.8 : 97.7)	94.8 (93.6 : 96.0)	0.021
<15% (normal range)	n	22	40	19		81	12	
>15%	n	0	0	0		0	0	
FIBTEM S A10 (n=105)	Mean (95% CI)	12.7 (11.6 : 13.9)	14.1 (12.6 : 15.7)	16.2 (14.6 : 17.8)	0.003	14.3 (13.3 : 15.3)	16.8 (13.5 : 20.1)	0.146
>7 (normal range 7-23)	n	24	43	24		91	13	
<7	n	1	0	0		1	0	
FIBTEM S MCF (n=105)	Mean (95% CI)	17.0 (13.1 : 20.9)	20.2 (16.9 : 23.5)	23.8 (19.8 : 27.7)	0.082	20.3 (18.0 : 22.5)	22.4 (13.4 : 31.3)	0.650
>9 (normal range 9-25)	n	24	42	24		90	13	
<9	n	1	1	0		2	0	

Table 6.3 ROTEM data grouped by day of illness and acute vs convalescent samples

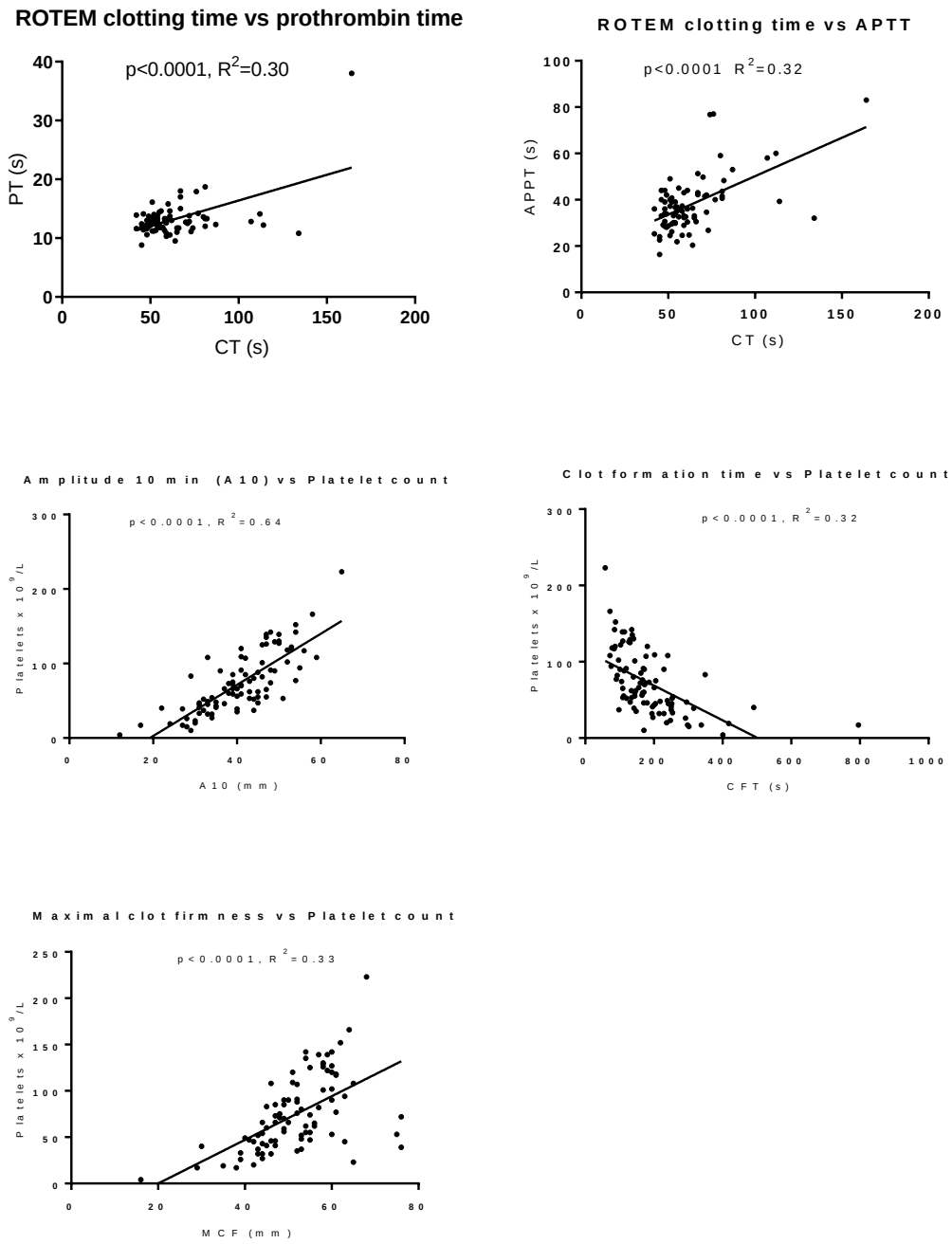


Figure 6.4 ROTEM and conventional coagulation testing correlations (all ROTEM timepoint measurements). P value from Pearson correlation.

6.4 Discussion

Crimean-Congo haemorrhagic fever is major emerging infectious threat and, although ribavirin is frequently utilised, the mainstay of case management is supportive care. Haemorrhage frequently occurs in severe disease and traditional laboratory tests (PT/APTT) do not show dramatic changes. This suggests that the coagulopathy seen in patients with CCHF is not due to disseminated intravascular coagulation (DIC) but may lie in areas not measured by conventional coagulation testing, such as a defect in platelets or fibrinolysis. This study is the first to investigate the use of a global haemostasis test, ROTEM, alongside conventional coagulation testing in CCHF, demonstrating that using ROTEM is feasible and can provide safe, rapid point of care results.

The analysis of this large cohort of patients in Turkey gives new insights into the aetiology and natural history of the coagulopathy in CCHF. Patients presented after a median of two days illness had a median platelet count of 73 x 10^9/L, with mean PT and APTT of 14 s and 38.3 s respectively, i.e. both coagulation tests were within the normal range of values, yet patients had a bleeding tendency. On admission most patients (42/45) had normal EXTEM S clotting times (CT), showing that initiation of clotting, thrombin formation and the start of clot polymerisation was not significantly abnormal in most cases at this point. In contrast, EXTEM S CFTs were abnormal in most cases at admission. CFT represents the time from initiation of clotting until a clot firmness of 20 mm is detected and elevated results indicate problems with fibrin polymerisation and stabilisation of the clot with Factor XIII and platelets. In the face of normal or slightly deranged APTT/PT, this points to a platelet and/or Factor XIII defect.

In keeping with the CFT results the MCF was abnormal in 21/45 patients on admission and was very deranged in 9/45. MCF represents the firmness of the clot, with abnormal values indicating reduced stabilisation of the clot by the polymerised fibrin, platelets and Factor XIII. Activation of fibrinolysis can be discounted as there was no significant reduction in MCF/clot lysis. In FIBTEM S, coagulation is activated as in EXTEM S but the addition of cytochalasin D, an antiplatelet agent, blocks the effects of platelets. The resulting clot formation therefore depends only on fibrin formation and fibrin polymerisation and this mainly relates to fibrinogen levels. The results show that the vast majority of patients at admission had normal FIBTEM S A10 (42/43) and MCF (41/43), indicating normal fibrinogen levels. In DIC there is consumption of coagulation factors with the formation of small thrombi throughout the vasculature leading to end organ failure. A key finding in DIC is the presence of hypofibrinogenaemia. The absence of significantly low fibrinogen levels or of activation of fibrinolysis are not in keeping with DIC.

A reversible coagulopathy was discovered in those who survived CCHF. Combined data from acute samples showed significant differences in all EXTEM S fields compared to those in

convalescent samples taken post discharge in a subset of survivors, which showed ROTEM results within normal ranges. When acute samples were stratified by day of illness, days 4-6 had the most abnormal values, indicating that this is the major coagulopathic period in CCHF with respect to a patient's ability to generate stable clots. Although there was no significant difference in median CT values, the highest percentage of abnormal CT results was also found in days 4-6. This is consistent with previous reports and consensus that patients with severe CCHF begin to haemorrhage in days 3-6.

In summary, these studies have demonstrated that a coagulopathy develops during CCHF infection, that is not associated with a major coagulation defect, as evidenced by only minor changes in PT, APTT, and ROTEM clotting time and FIBTEM. Importantly, ROTEM has shown no evidence of hyperfibrinolysis. Such a defect could be due to either Factor XIII deficiency or a platelet dysfunction, to account for the bleeding and the defect in clot firmness. Factor XIII merits further investigation but is unlikely to be the cause of the bleeding defect. The clinical pattern of bleeding is also in keeping with a platelet defect, as bleeding was predominantly from mucous membranes. This was not simply due to thrombocytopenia either, as most patients had platelet counts greater than 50 x 10^9/L and such counts are not usually associated with spontaneous bleeding.

Platelet dysfunction has been demonstrated in other viral haemorrhagic fevers, particularly by Cummins et al, who found a clear platelet function defect using platelet aggregometry in both Lassa and Argentinian HFs [184,228]. They may have similar pathogenic pathways as acute severe viral infections that produce similar patterns of bleeding. There is a small possibility that the platelet defect is due to a change in von Willebrand factor, possibly through loss of large molecular weight forms. This is unlikely, as this is usually either due to high shear stresses or an antibody against von Willebrand factor and has never been seen associated with a viral disease. This study excludes DIC as the cause of the bleeding defect in all patients at presentation with CCHF. In DIC there is a consumptive coagulopathy associated with low platelet counts (which were found), low FIBTEM values and prolonged PT and APTT, with prolongation of CT, all reflecting low coagulation factors: these were not found in this study. However, as highlighted by the second fatal case of CCHF described, DIC can occur in fatal cases of CCHF with multi-organ failure.

A limitation of the study is that less patients with severe/fatal disease were recruited than expected, but it demonstrated that the coagulopathy occurred in those graded with mild disease. The clinical and laboratory data are consistent with larger cohorts in Turkey in which PT prolongation occurred in only 14/404 patients and APTT >70 seconds was found in 15/404 patients [152]. CCHF severity scores were developed to predict mortality and have been validated

in Turkish healthcare settings where there is access to good supportive care and blood transfusion. In this study some participants had data missing at admission for severity score calculation and this potentially risked underscoring some participants. However, imputation in with maximum/minimum scores did not change the severity grouping of most participants or alter the significance of the results. Prognostic scoring at baseline is challenging and existing scoring systems require laboratory variables which are not routinely available in all settings. In routine practice, simple parameters such as a platelet count <50 x 10^9/L is used as the trigger for referral to tertiary centres, indicating more severe disease, increased likelihood of haemorrhage and the possible need for intervention with blood transfusion [160].

Although prospective, given the observational nature of the study it is not appropriate to analyse data stratified by subsequent ribavirin use. For similar reasons it is not possible to analyse the data on blood component therapy use in relation to ROTEM parameters in this study. The decision to use blood components was up to the treating clinicians with resulting variability in practice. ROTEM analysis was also not routinely undertaken at specific time points after transfusion to allow evaluation of effect on haemostasis, and this should be evaluated in future studies. A limitation of ROTEM is that it does not reflect what happens in the blood vessels due to its lack of being able to reproduce the endothelium. Serial daily ROTEM analysis of all cases would provide more complete data, but this study was limited by the practical challenges of time required for analysis, high caseloads and safety considerations. Earlier recruitment of patients during the illness course would have provided more data on the progression of the coagulopathy, but was constrained by delays in patient presentation and the time required for participant recruitment/informed consent.

The management of CCHF is challenging due to limited therapeutics available and its appreciable mortality. This ROTEM and conventional coagulation analysis has demonstrated that CCHF-related coagulopathy is predominantly related to clot development/stabilisation that is most marked during days 4-6 of the illness and in severe disease. In the context of normal/only slightly deranged coagulation screens and FIBTEM results, and combined with the absence of hyperfibrinolysis, this study provides the first evidence that the initial coagulopathy in CCHF probably relates to platelet dysfunction. Further studies are required to confirm these findings and look at possible mechanism(s).

Chapter 7: Cytokine changes in CCHF

7.1 Introduction

Following CCHFV infection the innate immune system comprising cellular and humoral responses act 'quasi-automatically' to generate an inflammatory response [70,71,229]. The key cells involved are monocytes/macrophages, neutrophils, eosinophils, basophils and natural killer cells. These leucocytes are activated by binding of pathogen-associated molecular patterns (PAMPs) molecules from CCHFV, or damage -associated molecular patterns (DAMPs) from necrotic cells, to pattern recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs)s, nucleotide-binding oligomerization domain-like (NOD-like) receptors, retinoic acid-inducible gene-I-like receptors (RIG-I-like) and C-type lectin receptors [70]. TLRs are the best studied and trigger a cascade of activation generating release of inflammatory cytokines.

7.1.1 Cytokine pathogenesis

Cytokines are a broad category of small proteins involved in cell signalling, that have a broad range of functions including immunomodulatory roles. They can be divided into several categories: interleukins; chemokines; interferons; tumour necrosis factor; and growth factors [70,111]. A tightly regulated cytokine network is crucial for elimination of invading pathogens and restricting excessive tissue damage [112,113], and sepsis has been shown to develop when this initial host response becomes amplified and deregulated [114–117]. Release of inflammatory cytokines induces new cytokine production and release, and this 'cytokine storm ' or 'cytokine cascade' is likely to be responsible for the many diverse and local effects of the 'sepsis syndrome' [70]. This has been demonstrated in a range of viral and bacterial infections, and was recently emphasised in the context of avian influenza [118]. Numerous pro-inflammatory cytokines have been identified during sepsis with IL-1β, IL-6, IL-12, and IL-17 being of crucial importance. However despite significant progress in the research of sepsis utilising the 'omics' (genomic, proteomic, metabolomic and transcriptomic approaches), therapeutic clinical trials targeting cytokines and the immune response in sepsis have failed [70]. The failure of immunomodulatory trials and the current lack of clear understanding about the organisation of host response reflects the fact that patients with sepsis are not a homogeneous group. Patients have different pathogens, presentations and levels of severity that make direct comparisons of pathophysiology and clinical trials focussed on immune response modification challenging.

7.1.2 Cytokines in viral haemorrhagic fevers/CCHF

A common pathogenic feature of VHFs is the ability of the virus to disable the host immune response by attacking and manipulating macrophages and dendritic cells. In severe cases of CCHF, it has been suggested that this, combined with the deregulation and excessive pro-inflammatory cytokine release, leads to toxic endothelium effects, increased vascular

permeability, multi-organ failure and shock [87]. A significant number of studies have evaluated the role of cytokines in CCHF, most frequently through analysis of baseline admission samples and direct comparison of severity groups or fatal/non-fatal disease. The majority of the 18 studies are from Turkey (14/18), with the largest study including 80 patients. Few have presented longitudinal data; one study of 31 patients evaluated a panel of 4 cytokines daily for 7 days, demonstrating that IL-6 and TNF-α were consistently higher in fatal cases. Another recent study of 52 patients evaluated a panel of cytokines every 3 days (119 samples in total), demonstrating that IL-6, IL-8, IL-10, IL-10/ 12, IFN-γ, MCP-1, and MIP-1β were higher in patients with CCHF than controls, and that IL-6 and IL-8 were higher in the first 5 days of illness in fatal cases. Evaluation of the data from these studies broadly demonstrates the that high serum levels of pro-inflammatory cytokines, particularly IL-6 and TNF-α, have been implicated as negative prognostic factors in CCHF and other VHFs [97,98,119,121–124]. IL-10 is an anti-inflammatory cytokine that has also been shown to be higher is severe/fatal disease in some CCHF studies [119,123,125], but this was not reproduced in other studies [120,126]. IL-12 production has demonstrated a mixed picture when assessing CCHF severity and comparing patients versus controls [97,121,126]. Other studies investigating endothelial adhesion mediators have shown VCAM-1 and sICAM-1 to be higher in severe cases [127,128], and other markers of endothelial dysfunction to be higher in haemorrhagic cases [129].

Overall the ability to draw prognostic inferences from these studies is usually limited by retrospective design, incomplete data sampling, small study numbers and the evaluation of cytokines at only one time point. Patients with CCHF present at different stages of illness and whilst baseline evaluation of cytokines may be useful to guide incorporation of these variables into prognostic severity scores, larger longitudinal studies are required to provide greater insights into the dynamic host immune response over time. Sample integrity for cytokine measurement is also important, and benefits from early processing and storage in ultra-low freezers. Repeated freeze-thaw cycles of legacy samples taken in different outbreaks may affect cytokine results and the ability to draw clear and valid conclusions. The ability to us multiplex assays in extremely beneficial due to the number of potential cytokines involved, the ability to use multiplex assays is extremely beneficial, but in common with other assays is technically difficult due to the highly pathogenic nature of VHFs.

Aims of cytokine analysis

1. Investigate the host immune response to clinical infection through serial measurement of cytokine/chemokine levels and quantitative serological response, related to disease severity and clinical outcomes.

2. Test the hypothesis that severe/fatal CCHF is associated with a deregulated cytokine network characterised by an exaggerated pro-inflammatory cytokine response (IL-1β, IL-6, IL-8 and tumour necrosis factor alpha [TNF-α], and reduced IL-12

7.2 Methods

After participant recruitment blood samples for multiplex cytokine analysis were taken daily from patients with confirmed CCHF, during their acute admission and at 14 and 30 days after onset of clinical disease. Blood was drawn into serum gel tubes, centrifuged and then serum removed and aliquoted into cryovials before storage at -70^{0}C. Priority for sample processing was given to serum samples and the time period from daily blood draw to freezing of serum was less than 4 hours. Samples were then transferred to PHE Porton at -70^{0}C. A commercial human 19-plex luminex kit was used and the assay was performed according to the manufacturer's instructions (Millipore,Watford, UK). All samples were run in duplicate.

Luminex staining

1. The wells of the 1.2-µmfilter membrane 96-well microtiter plates were pre-wetted with assay buffer.
3. 25 µl of sample, standard and quality control preparations were added to the relevant wells and incubated with pre-mixed microbeads for 2h on an orbital plate shaker at room temperature.
4. The plates were washed twice with assay wash buffer and 25 µl biotinylated detector antibody added per well.
5. Samples were incubated for 1h at room temperature on the plate shaker. Without washing, 25 µl/well streptavidin–phycoerythrin solution was added, and plates incubated for a further 30min at room temperature on a plate shaker, protected from direct light.

Microbead fixation and analysis of results

1. After completion of staining, the microbeads were washed twice with assay wash buffer.
2. Beads were then left overnight (17h) with 100 µl/well of 10% formalin. 10% formalin was made by diluting 100% formalin (40% w/v formaldehyde solution) (Scientific Laboratory Supplies, Nottingham, England) 1:10 with PBS.
3. Before analysing, microbeads were washed twice in assay wash buffer and resuspended in 100 µl/well of luminex sheath fluid.

Analysis of results

1. The luminex assay was acquired on a luminex-200™ instrument using Exponent software (Invitrogen, Paisley, England).

2. An acquisition gate of between 8000 and 13,500 was set to discriminate against any doublet events and ensure that only single microbeads were measured.
3. 50 events per region were collected and the median fluorescence intensity (MFI) measured.
4. MFI were converted to concentrations using results from a standard cytokine preparation.
5. The cytokine standard was diluted 1:4 with a starting concentration of 10,000 pg/ml, giving a lower limit of detection of 3.2 pg/ml.

7.3 Results - analysis and stratification of cytokine results

Cytokines were analysed at admission, daily across the course of acute admission and during follow-up in convalescence. Means (standard deviation or 95% confidence interval (CI)) or medians (interquartile ranges) were utilised as appropriate for continuous variables, with distribution tested for normality by the Kolmogorov-Smirnov and Shapiro-Wilk Tests. Continuous variables measured longitudinally were compared using linear regression models. Log-transformed viral load data and cytokines levels were compared by Pearson correlation analysis. Heatmaps were generated using Morpheus software (https://software.broadinstitute.org/morpheus/). No imputation for missing data was made due to small sample sizes. Hypothesis tests were two-tailed ($p<0{\cdot}05$) and analyses were performed using the SPSS (version 24). CCHF cases were stratified for cytokine comparison according to disease severity at baseline, lowest platelet count during admission (plts <50 x 10^9/L or >50 x 10^9/L), haemorrhage during admission and outcome.

1. Cytokines at admission vs 30-day cytokine levels
2. Cytokines at admission by severity (mild vs moderate/severe disease)
3. Cytokines at admission by lowest platelet count (platelet count <50 x 10^9/L or >50 x 10^9/L)
4. Cytokines at admission by outcome
5. Serial cytokines : all; severity (mild vs moderate/severe); platelet count (plts <50 x 10^9/L vs > 50x 10^9/L); and haemorrhage.
6. Linear regression: all; severity (mild vs moderate/severe); platelet count (plts <50 x 10^9/L vs >50 x 10^9/L); and fatal/survived
7. Correlation cytokines and CCHF log viral load.

No.	Cytokine	Produced by	Function
Pro-Inflammatory			
1.	IL-6 Interleukin-6	T cells Macrophages	ICAM/VCAM upregulated on endothelial cells Upregulates M-CSF receptor expression Can increase Il-10 expression Inhibits TNF-α and IL-1
2.	IL-1B Interleukin-1b (catabolin)	Macrophages Monocytes Endothelial cells	Induces synthesis of IL-6, IL8. MCP-1
3.	TNF-α Tumour necrosis factor alpha	Macrophages mainly T and B Cells Monocytes Mast cells NK cells Endothelial cells	Induces synthesis of IL-1, IL-6 Stimulate acute phase proteins Activates endothelial cells and neutrophils
4.	IFN-γ Inteferon-gamma	T cells NK cells	Activates macrophages, cytotoxic t cells and NK cells Increase differentiation of Th1 lymphocytes Increase class 1 and II MHCs Regulated by IL-12/IL-18 Inhibited by IL-4/IL-10
5.	IFN- α2 Interferon- a2		Activates NK cells Induces anti-viral state
6.	IL-17A	Th2 cells	Induces synthesis of IL-6, GM-CSF, IL-B, TNF, IL-8, MCP-1
7.	IL-5	Th2 cells	B-lymphocytes – proliferation and differentiation Eosinophils – proliferation and activation
8.	IL-2	T-cells	Promotes clonal expansion of T and B lymphocytes NK cells proliferation and activation
9.	IL-12/p70	Dendritic cells Macrophages	T-cell Stimulating factor stimulates IFN-γ and TNF-α release from T-cells Induces Th1 differentiation from naive T-cells Reduces IL-4 Activates NK cells Also has anti-inflammatory /regulatory functions
10.	IL-15		Similar to IL-2 - Proliferation of T and B lymphocytes and NK cells
11.	G-CSF Granulocyte colony stimulating factor	Endothelium macrophages	Growth and differentiation of neutrophils
12.	GM-CSF Granulocyte macrophage	Th- cells	Growth and differentiation of monocytes and dendritic cells

	colony stimulating factor		
Regulatory/anti-inflammatory cytokines			
13.	IL-10	Th2 cells Macrophages Mast cells Cytotoxic t cells	Key immuno-regulatory /anti-inflammatory cytokine Human cytokine inhibitory factor Inhibit synthesis of IFN-γ, IL-2, IL-3, IL-6, TNF-α, GM-CSF, Il-12and IL-17
14.	IL-4	Macrophages Th2 cells	Regulatory/ant-inflammatory Promotes proliferation of B and T cells Stimulates development and growth of differentiated Th2 cells from naïve Th cells Suppresses production of TH1 cells Increases expression of VCAM-1 and decreases E-selectin
Chemokines (pro-inflammatory)			
15	IL-8 /CXCL8 chemokine	Macrophages Endothelial cells	Neutrophil chemotactic factor – promotes chemotaxis and degranulation
16	IP-10/CXCL10 Interferon-gamma-inducible protein 10	Monocytes/macrophages, plus endothelial cells and fibroblasts	Chemoattraction macrophages, T cells and NK cells
17	MCP-1/CCL2	Monocytes/macrophages plus endothelial cells, and fibroblasts	Monocyte chemoattractant protein Attracts monocytes, memory T-cells and dendritic cells
18	MIP-1α/CCL3 Macrocyte inflammatory protein alpha /chemokine ligand 3	Macrophages	Induce synthesis of IL-5, IL-6 and TNF-α, Pyrogen
19	MIP-1β/CCL4 Macrocyte inflammatory protein alpha/Chemokine ligand 4	Lymphocytes	Chemoattraction of NK cells, monocytes

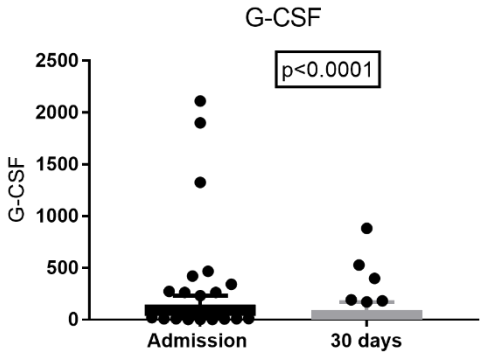
Table 7.1 Cytokines/chemokines evaluated in the 19 plex panel

7.3.1 Cytokines at admission vs 30 days

At admission 102/104 participants had cytokines levels measured and 60/104 participants attended follow-up at 30 days. There was no significant difference in age, sex or severity ratio of the 60 participants that attended follow-up in comparison to the 102 participants at admission. The distribution of all of the cytokine levels was significantly higher at admission compared to 30 days part from in IL-17A, IL-1β, IL-2 and IL-5.

Cytokine (pg/ml)	Admission (n=102)	30 days (n=60)	p-value
G-CSF	83.3 (37.1-144.9)	21.2 (3.2-94.8)	**<0.0001**
GM-CSF	8.5 (4.8-15.5)	3.2 (3.2-8.6)	**0.002**
IFN- α2	53.0 (18.0-133.0)	3.2 (3.2-3.3)	**<0.0001**
IFN-γ	17.5 (9.5-31.7)	6.7 (3.2-18.7)	**<0.0001**
IL-10	118.7 (38.7-288.9)	3.2 (3.2-4.6)	**<0.0001**
IL-12	3.2 (3.2-8.2)	3.2 (3.2-13.2)	**<0.0001**
IL-15	8.8 (4.1-20.1)	3.2 (3.2-3.2)	**<0.0001**
IL-17A	4.0 (3.2-9.5)	3.2 (3.2-15.8)	0.90
IL-1β	3.2 (3.2-3.2)	3.2 (3.2-3.9)	0.42
IL-2	3.2 (3.2-3.2)	3.2 (3.2-3.2)	0.87
IL-4	3.2 (3.2-6.3)	3.2 (3.2-3.2)	**0.004**
IL-5	3.2 (3.2-3.2)	3.2 (3.2-3.2)	0.39
IL-6	9.6 (3.2-26.9)	3.2 (3.2-4.8)	**<0.0001**
IL-8	34.9 (21.3-73.0)	19.4 (7.0-29.5)	**<0.0001**
IP-10	8426.0 (4777.6-13835.0)	495.6 (273.2-741.4)	**<0.0001**
MCP-1	1435.1 (799.6-2688.7)	555.2 (429.4-756.0)	**<0.0001**
MIP-1α	18.5 (9.3-28.0)	7.6 (3.2-20.0)	**0.0004**
MIP-1β	35.2 (25.4-58.4)	23.5 (12.8-39.2)	**<0.0001**
TNF-α,	24.5 (17.0-46.2)	9.1 (6.4-13.8)	**<0.0001**

Table 7.2 Cytokine levels (median/IQR) at admission and at 30 days after onset of symptoms. P-values calculated by Mann-Whitney U Test with statistically significant results ($p<0.05$) highlighted in bold.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G-CSF
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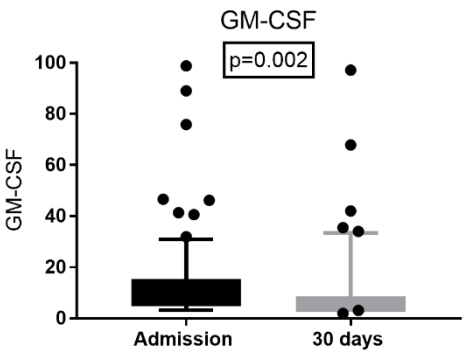
Admission
30 days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20
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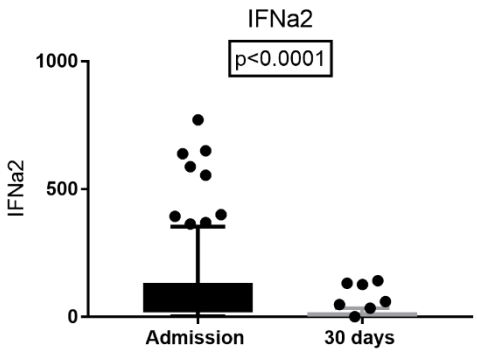
Admission
30 days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IFNa2
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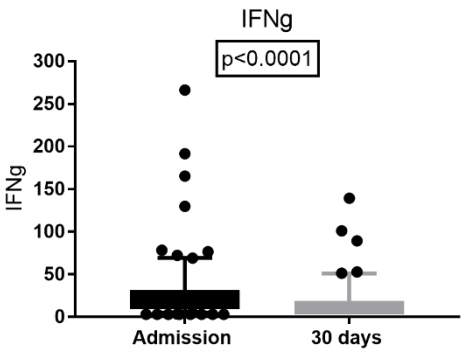
500
0
Admission
30 days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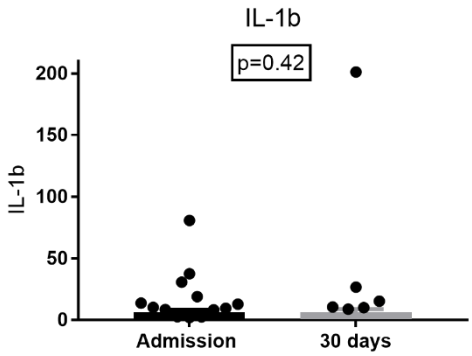
50
0
Admission
30 days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IL-1b
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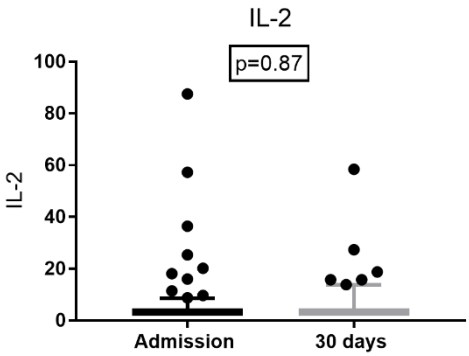
Admission
30 days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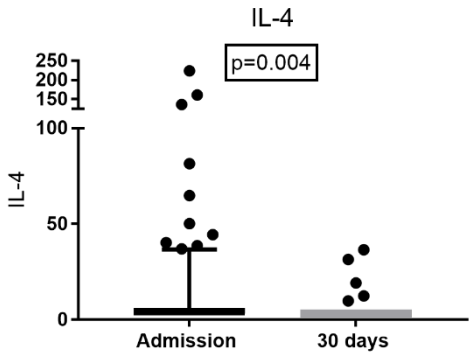
Admission
30 days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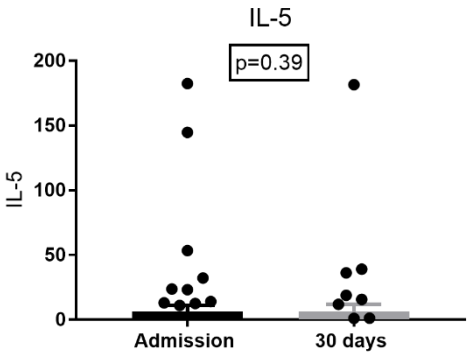
Admission
30 days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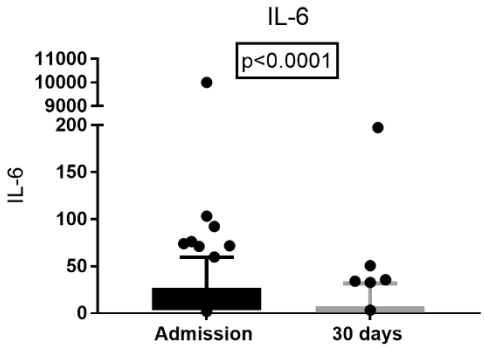
Admission
30 days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Admission
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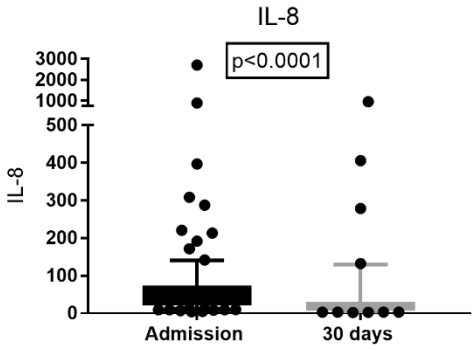
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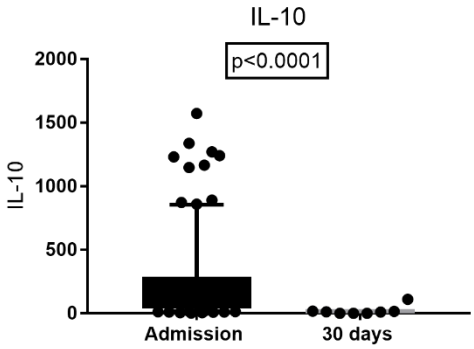
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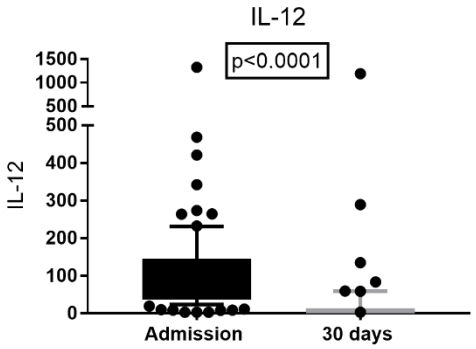
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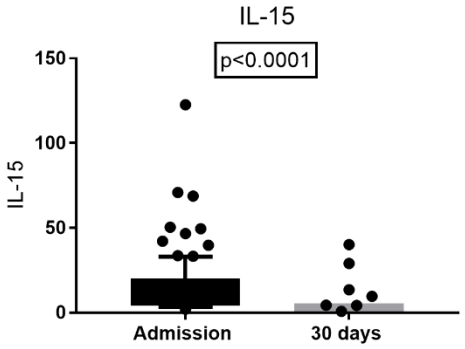
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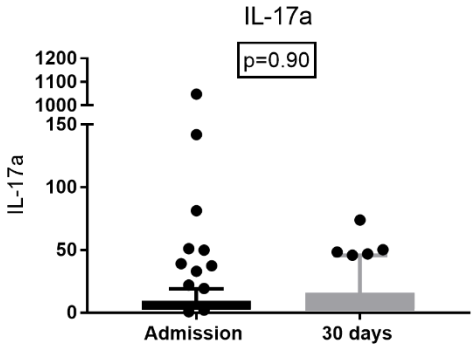
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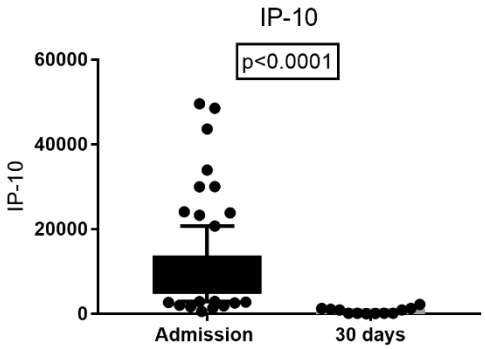
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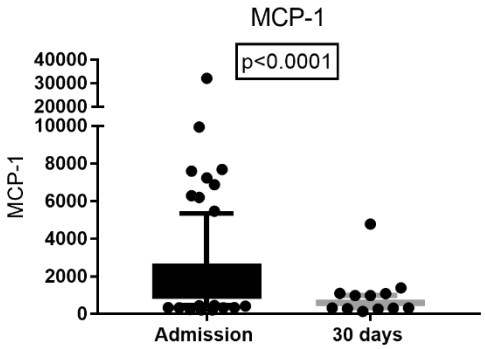
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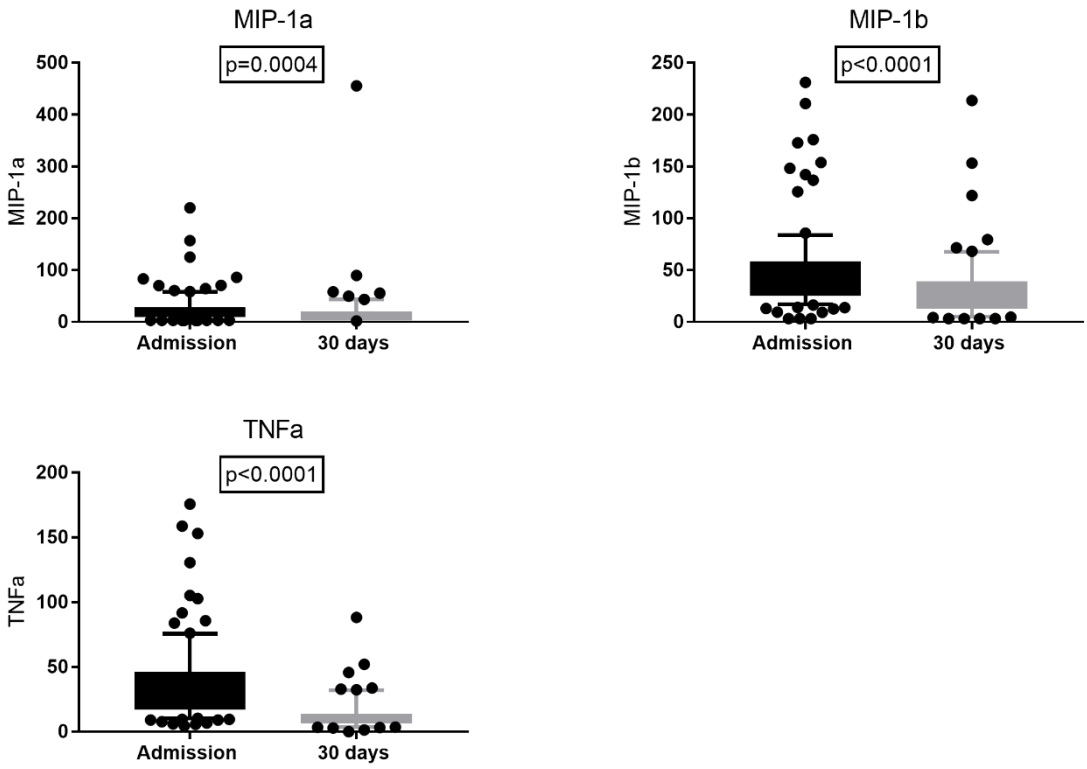


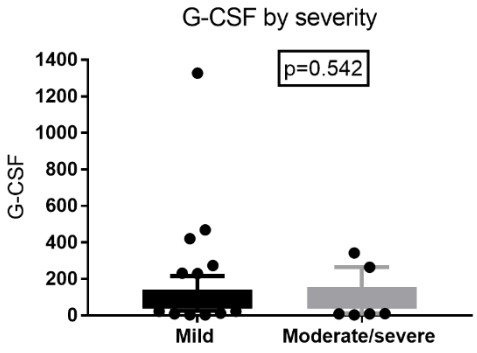
Figure 7.1 Cytokine levels at admission compared to 30 days after onset of symptoms. (Y axis pg/ml) Boxplots (whiskers 10-90th centile) with outliers. P-values calculated by Mann-Whitney U Tests.

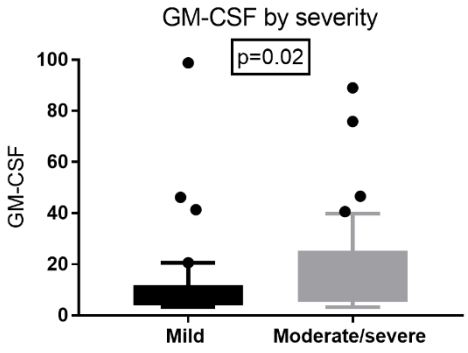
7.3.2 Cytokines levels at admission by disease severity

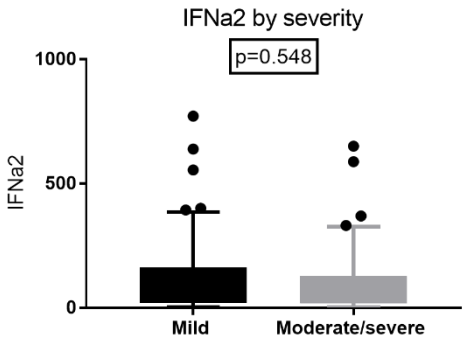
At admission cytokines were measured in 102/104 of participants at a median of 4 days (IQR 3-6 days) after onset of symptoms. There were significant differences in the distribution of cytokine levels between mild and moderate/severe groups in GM-CSF (p=0.024), IL-10 (P<0.0001), IL-12 (p=0.022), IL-17A (p=0.013), IL-8 (p=0.009) and IP-10 (p=0.015), with moderate/severe group demonstrating higher cytokine levels (Table 7.3, Figure 7.2).

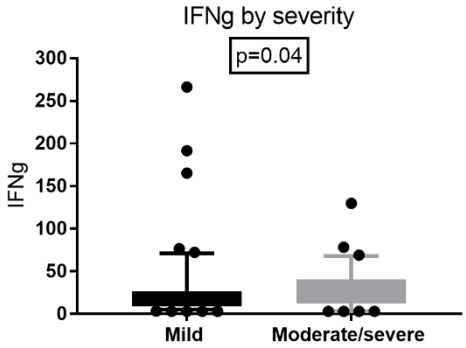
Cytokine (pg/ml)	All (n=102)	Mild (n=62)	Moderate /severe (n=40)	**p-value**
G-CSF	83.3 (37.1-144.9)	82.1 (37.1-140.7)	90.1 (36.3-156.3)	0.542
GM-CSF	8.5 (4.8-15.5)	7.1 (4.0-11.9)	11.7 (5.3-25.3)	**0.024**
IFN-α2	53.0 (18.0-133.0)	57.4 (19.2-161.5)	42.3 (17.9-127.4)	0.548
IFN-γ	17.5 (9.5-31.7)	13.6 (9.1-26.4)	20.6 (12.6-40.6)	0.044
IL-10	118.7 (38.7-288.9)	77.4 (32.5-178.7)	280.7 (94.5-843.7)	**<0.0001**
IL-12	3.2 (3.2-8.2)	3.2 (3.2-6.1)	4.8 (3.2-14.1)	**0.022**
IL-15	8.8 (4.1-20.1)	8.4 (3.7-17.6)	9.9 (4.6-28.3)	0.376
IL-17A	4.0 (3.2-9.5)	3.2 (3.2-5.9)	6.0 (3.2-15.0)	**0.013**
IL-1β	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-3.3)	0.877
IL-2	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-4.1)	0.112
IL-4	3.2 (3.2-6.3)	3.2 (3.2-3.2)	3.2 (3.2-14.0)	0.031
IL-5	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-3.2)	0.313
IL-6	9.6 (3.2-26.9)	8.3 (3.2-21.1)	9.8 (4.7-34.6)	0.113
IL-8	34.9 (21.3-73.0)	31.3 (20.9-53.0)	54.2 (24.9-131.6)	**0.009**
IP10	8426.0 (4777.6-13835.0)	7320.3 (4395.5-11443.3)	10000.0 (5875.9-18215.0)	**0.015**
MCP1	1435.1 (799.6-2688.7)	1356.4 (870.4-2290.6)	1587.2 (674.7-3543.7)	0.661
MIP-1α	18.5 (9.3-28.0)	16.0 (9.3-25.0)	20.9 (9.2-37.2)	0.088
MIP-1β	35.2 (25.4-58.4)	34.6 (23.1-58.4)	37.1 (25.9-61.2)	0.810
TNF-α,	24.5 (17.0-46.2)	21.0 (16.8-35.6)	35.2 (17.1-52.3)	0.067

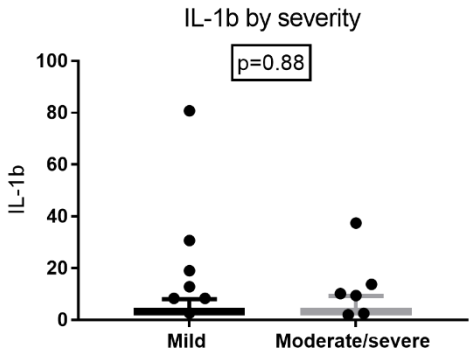
Table 7.3 Cytokines levels (median, IQR) at admission by grouped disease severity. p-values calculated by Mann-Whitney U Test with statistically significant results (p<0.05) highlighted in bold

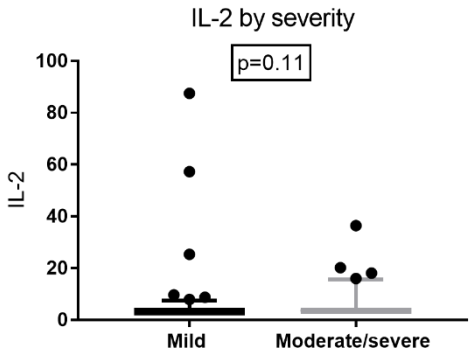

G-CSF by severity
p=0.542
G-CSF
1400
1200
1000
800
600
400
200
0
Mild
Moderate/severe

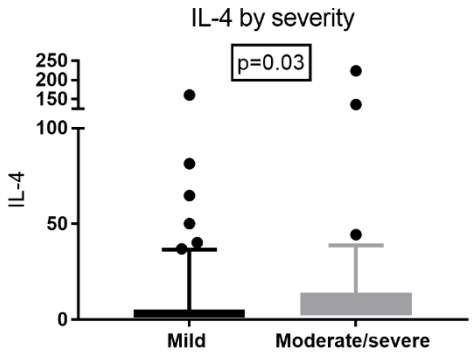

GM-CSF by severity
p=0.02
GM-CSF
100
80
60
40
20
0
Mild
Moderate/severe

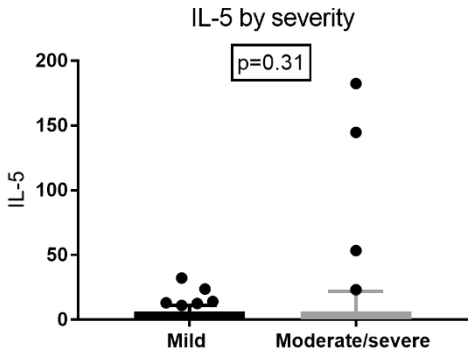

IFNa2 by severity
p=0.548
IFNa2
1000
500
0
Mild
Moderate/severe

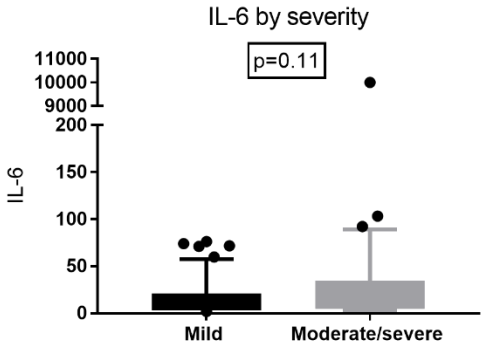

IFNg by severity
p=0.04
IFNg
300
250
200
150
100
50
0
Mild
Moderate/severe

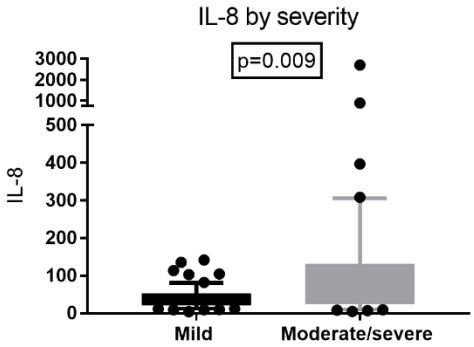

IL-1b by severity
p=0.88
IL-1b
100
80
60
40
20
0
Mild
Moderate/severe

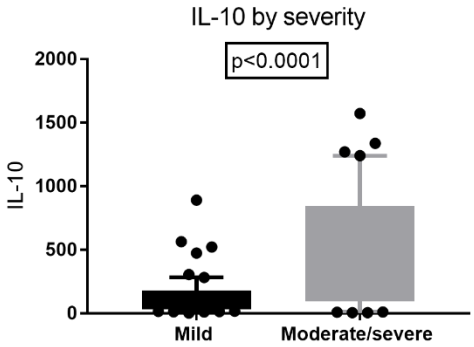

IL-2 by severity
p=0.11
IL-2
100
80
60
40
20
0
Mild
Moderate/severe

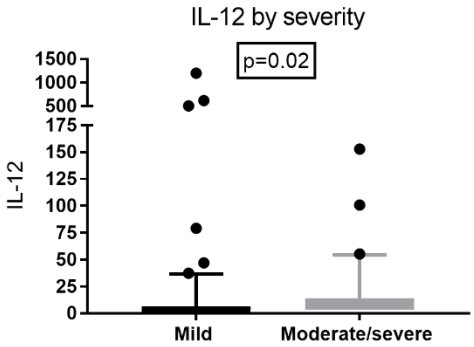

IL-4 by severity
p=0.03
IL-4
250
200
150
100
50
0
Mild
Moderate/severe

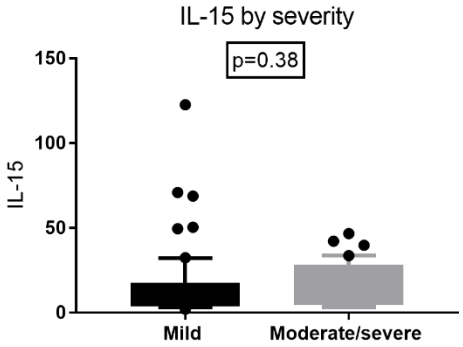

IL-5 by severity
p=0.31
IL-5
200
150
100
50
0
Mild
Moderate/severe

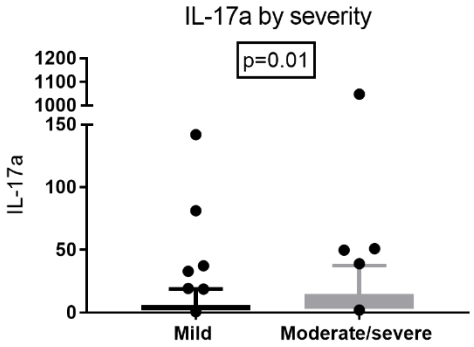

IL-6 by severity
p=0.11
IL-6
11000
10000
9000
200
150
100
50
0
Mild
Moderate/severe

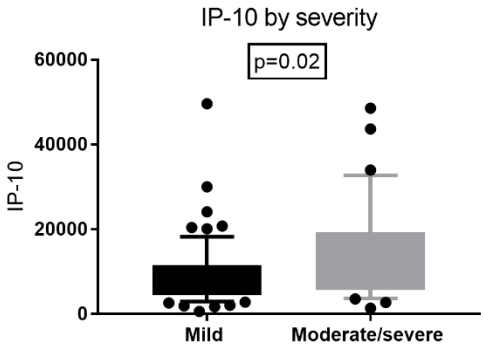

IL-8 by severity
p=0.009
IL-8
3000
2000
1000
500
400
300
200
100
0
Mild
Moderate/severe

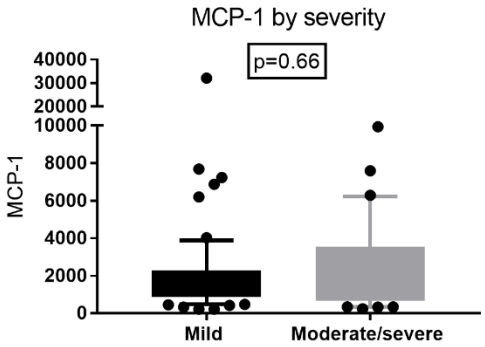

IL-10 by severity
p<0.0001
IL-10
2000
1500
1000
500
0
Mild
Moderate/severe


IL-12 by severity
p=0.02
IL-12
1500
1000
500
175
150
125
100
75
50
25
0
Mild
Moderate/severe


IL-15 by severity
p=0.38
IL-15
150
100
50
0
Mild
Moderate/severe


IL-17a by severity
p=0.01
IL-17a
1200
1100
1000
150
100
50
0
Mild
Moderate/severe


IP-10 by severity
p=0.02
IP-10
60000
40000
20000
0
Mild
Moderate/severe


MCP-1 by severity
p=0.66
MCP-1
40000
30000
20000
10000
8000
6000
4000
2000
0
Mild
Moderate/severe

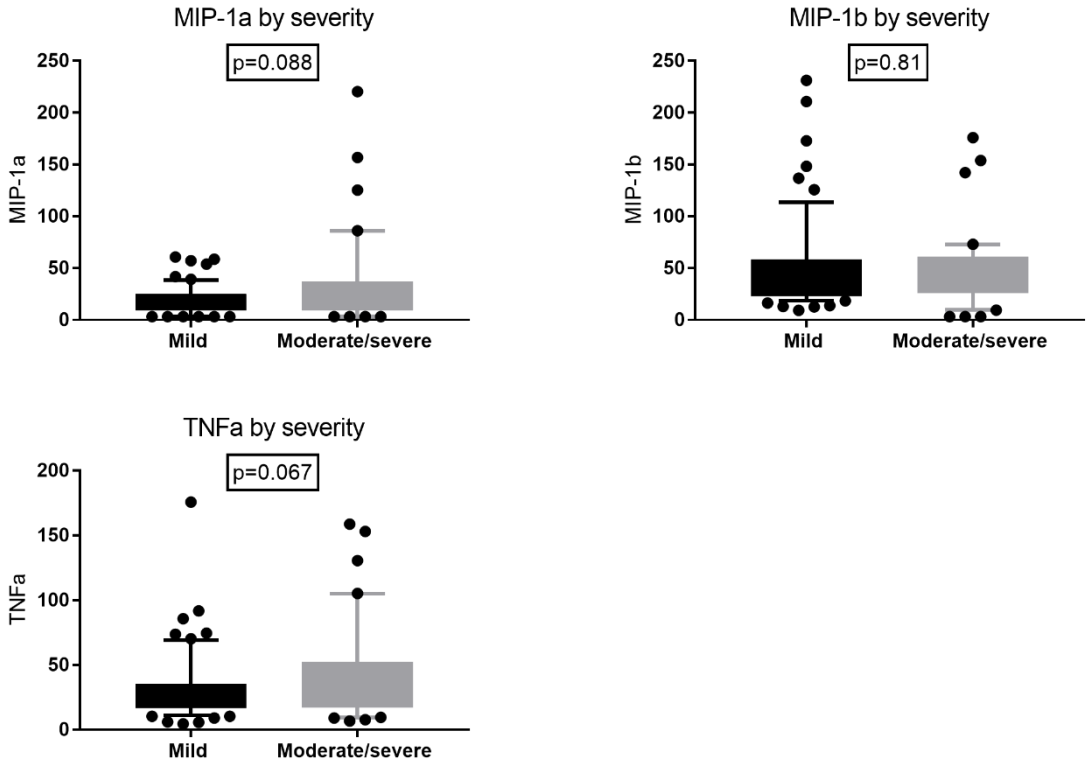


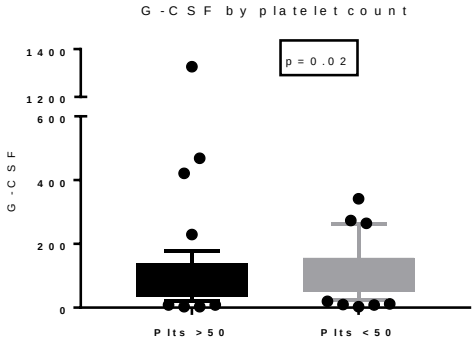
Figure 7.2 Cytokines at admission by severity (y axis pg/ml). Boxplots (whiskers 10-90th centile) with outliers. p-values calculated by Mann-Whitney U Test

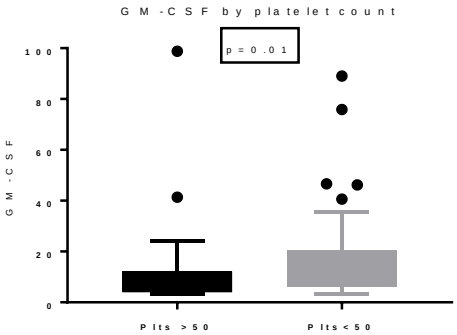
7.3.3 Cytokines levels at admission by lowest platelet count (plts <50 x 10^9/L or >50 x 10^9/L)

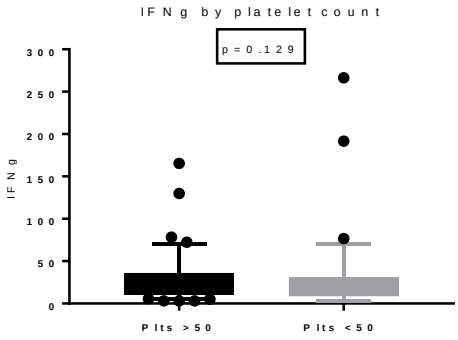
There were significant differences in distribution of cytokines at admission when groups were stratified by lowest platelet count recorded during the course of admission (platelet count <50 x 10^9/L or >50 x 10^9/L). Higher median levels of G-CSF (p=0.02), GM-CSF (p=0.01), IFN-A2 (p=0.01), IL-10 (p=0.02), IL-15 (p=0.001), IL-6 (p=0.001), IL-8 (p<0.0001), IP-10 (p=<0.0001), MCP-1 (p=0.001), MIP-1α (p=0.001), MIP-1β (p=0.006) and TNF-α, (p=0.002) observed in the group that had developed a platelet count of <50 x 10^9/L.

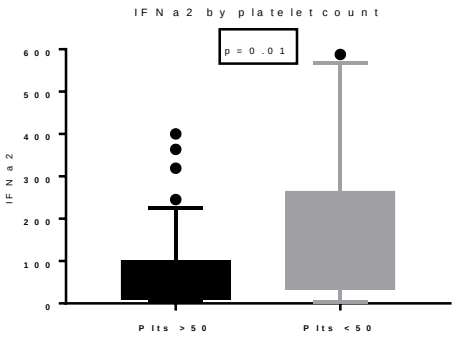
Cytokine	All (n=102)	Plts >50 x 10^9/L (n=47)	Plts <50 x 10^9/L (n=55)	p-value
G-CSF	83.3 (37.1-144.9)	55.3 (34.4-139.4)	96.1 (50.5-156.7)	**0.02**
GM-CSF	8.5 (4.8-15.5)	6.2 (4.0-12.1)	11.1 (6.4-20.6)	**0.01**
IFN-α2	53.0 (18.0-133.0)	39.9 (9.4-102.2)	86.5 (32.2-265.6)	**0.01**
IFN-γ	17.5 (9.5-31.7)	13.4 (8.7-30.9)	18.7 (10.3-36.1)	0.13
IL-10	118.7 (38.7-288.9)	82.3 (29.5-225.8)	145.2 (50.6-436.5)	**0.02**
IL-12	3.2 (3.2-8.2)	3.2 (3.2-6.4)	3.2 (3.2-16.0)	0.137
IL-15	8.8 (4.1-20.1)	5.8 (3.2-12.9)	15..0 (6.8-27.4)	**0.001**
IL-17A	4.0 (3.2-9.5)	3.2 (3.2-3.2)	4.7 (3.2-9.5)	0.39
IL-1β	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-3.2)	0.57
IL-2	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-3.4)	0.39
IL-4	3.2 (3.2-6.3)	3.2 (3.2-3.2)	3.2 (3.2-10.7)	0.20
IL-5	3.2 (3.2-3.2)	3.2 (3.2-3.2)	3.2 (3.2-3.2)	0.59
IL-6	9.6 (3.2-26.9)	4.9 (3.2-13.4)	16.8 (5.3-40.4)	**0.0001**
IL-8	34.9 (21.3-73.0)	28.1 (18.1-47.1)	55.3 (29.9-113.4)	**<0.0001**
IP-10	8426.0 (4777.6-13835.0)	5260.5 (3223.9-7779.9)	10000.0 (6722.0-16593.0)	**<0.0001**
MCP-1	1435.1 (799.6-2688.7)	993.54 (663.4-1825.0)	1860.6 (1043.0-3722.0)	**0.001**
MIP-1α	18.5 (9.3-28.0)	12.5 (7.8-20.9)	24.3 (14.9-37.3)	**0.001**
MIP-1β	35.2 (25.4-58.4)	31.4 (21.8-41.1)	43.4 (28.5-67.3)	**0.006**
TNF-α	24.5 (17.0-46.2)	19.8 (16.4-30.6)	37.6 (20.2-66.4)	**0.002**

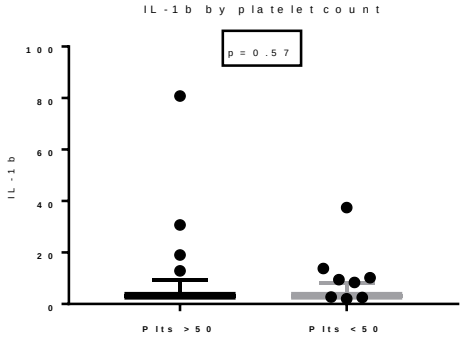
Table 7.4 Cytokines levels (median, IQR) at admission grouped by lowest platelet count. P-values calculated by Mann-Whitney U Test with statistically significant results (p<0.05) highlighted in bold.

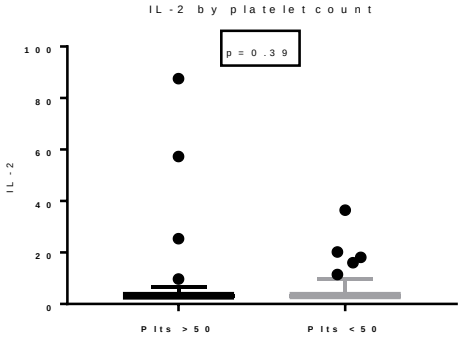

G-CSF by platelet count
p = 0.02
G-CSF
1400
1200
600
400
200
0
Plts >50
Plts <50

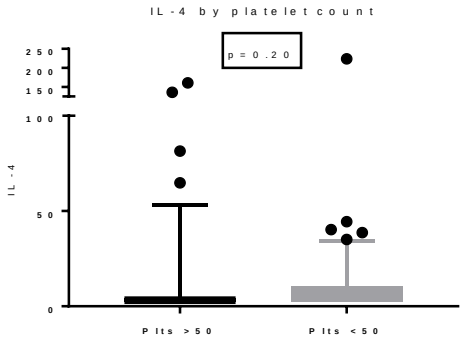

GM-CSF by platelet count
p = 0.01
GM-CSF
100
80
60
40
20
0
Plts >50
Plts <50

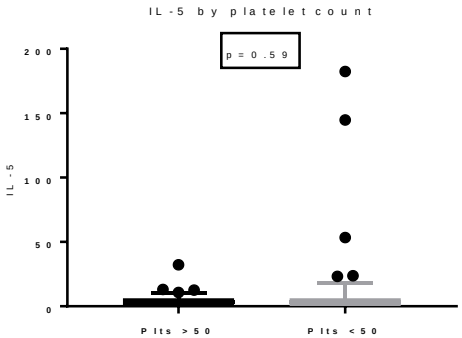

IFNg by platelet count
p = 0.129
IFNg
300
250
200
150
100
50
0
Plts >50
Plts <50

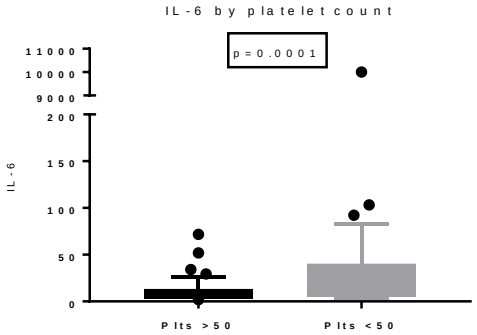

IFNa2 by platelet count
p = 0.01
IFNa2
600
500
400
300
200
100
0
Plts >50
Plts <50

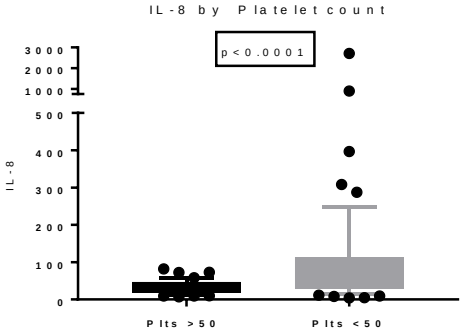

IL-1b by platelet count
p = 0.57
IL-1b
100
80
60
40
20
0
Plts >50
Plts <50

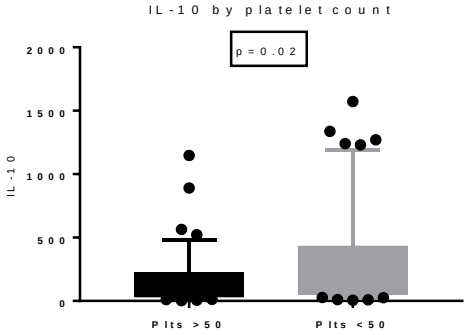

IL-2 by platelet count
p = 0.39
IL-2
100
80
60
40
20
0
Plts >50
Plts <50

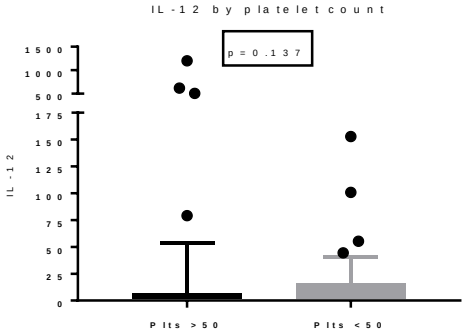

IL-4 by platelet count
p = 0.20
IL-4
250
200
150
100
50
0
Plts >50
Plts <50

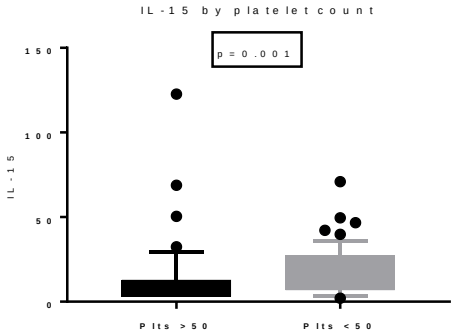

IL-5 by platelet count
p = 0.59
IL-5
200
150
100
50
0
Plts >50
Plts <50

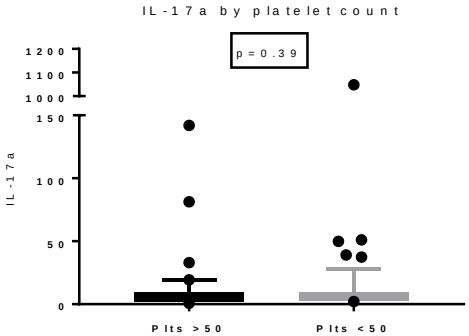

IL-6 by platelet count
p = 0.0001
IL-6
Plts >50
Plts <50

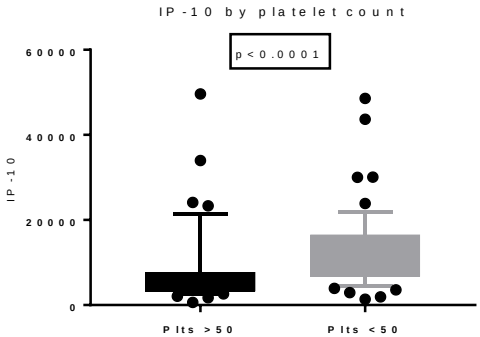

IL-8 by Platelet count
p < 0.0001
IL-8
Plts >50
Plts <50

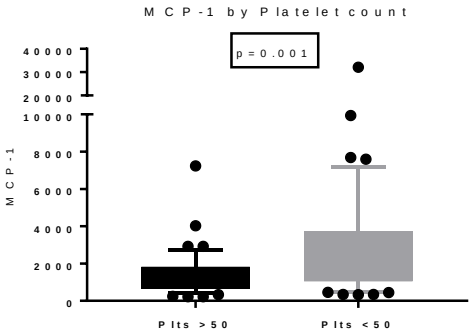

IL-10 by platelet count
p = 0.02
IL-10
Plts >50
Plts <50


IL-12 by platelet count
p = 0.137
IL-12
Plts >50
Plts <50


IL-15 by platelet count
p = 0.001
IL-15
Plts >50
Plts <50


IL-17a by platelet count
p = 0.39
IL-17a
Plts >50
Plts <50


IP-10 by platelet count
p < 0.0001
IP-10
Plts >50
Plts <50


MCP-1 by Platelet count
p = 0.001
MCP-1
Plts >50
Plts <50

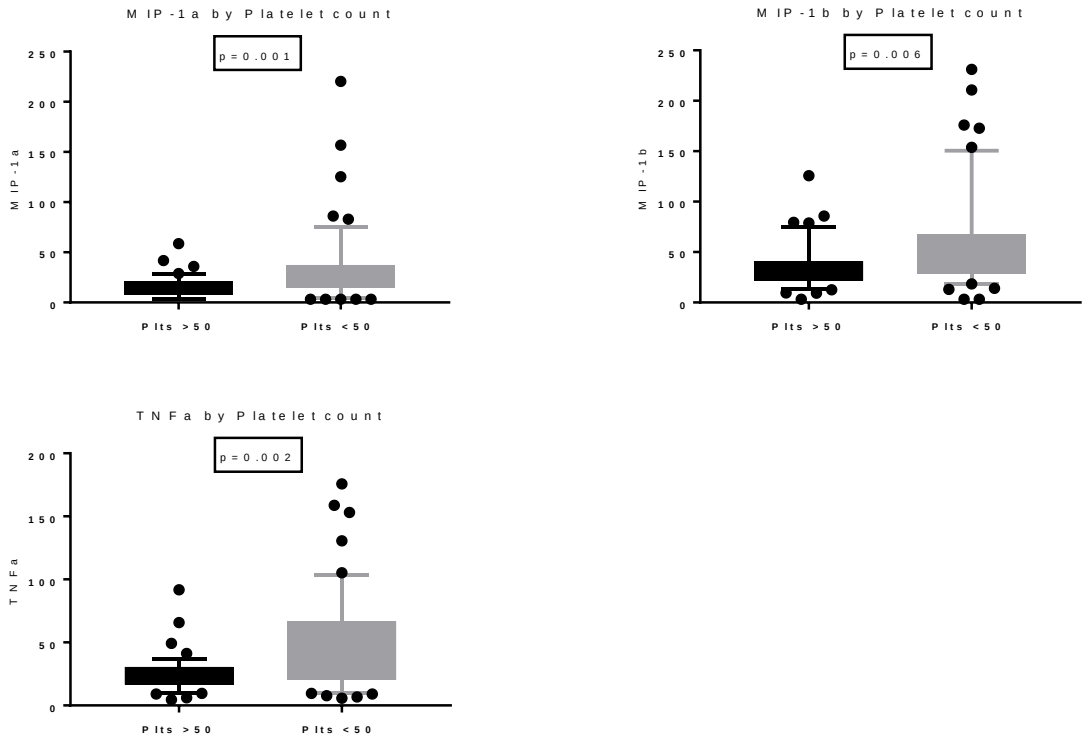


Figure 7.3 Cytokine levels at admission by lowest platelet count (x 10^9/L) (y axis pg/ml). Boxplots (whiskers 10-90th centile) with outliers. p-values calculated by Mann-Whitney U Tests.

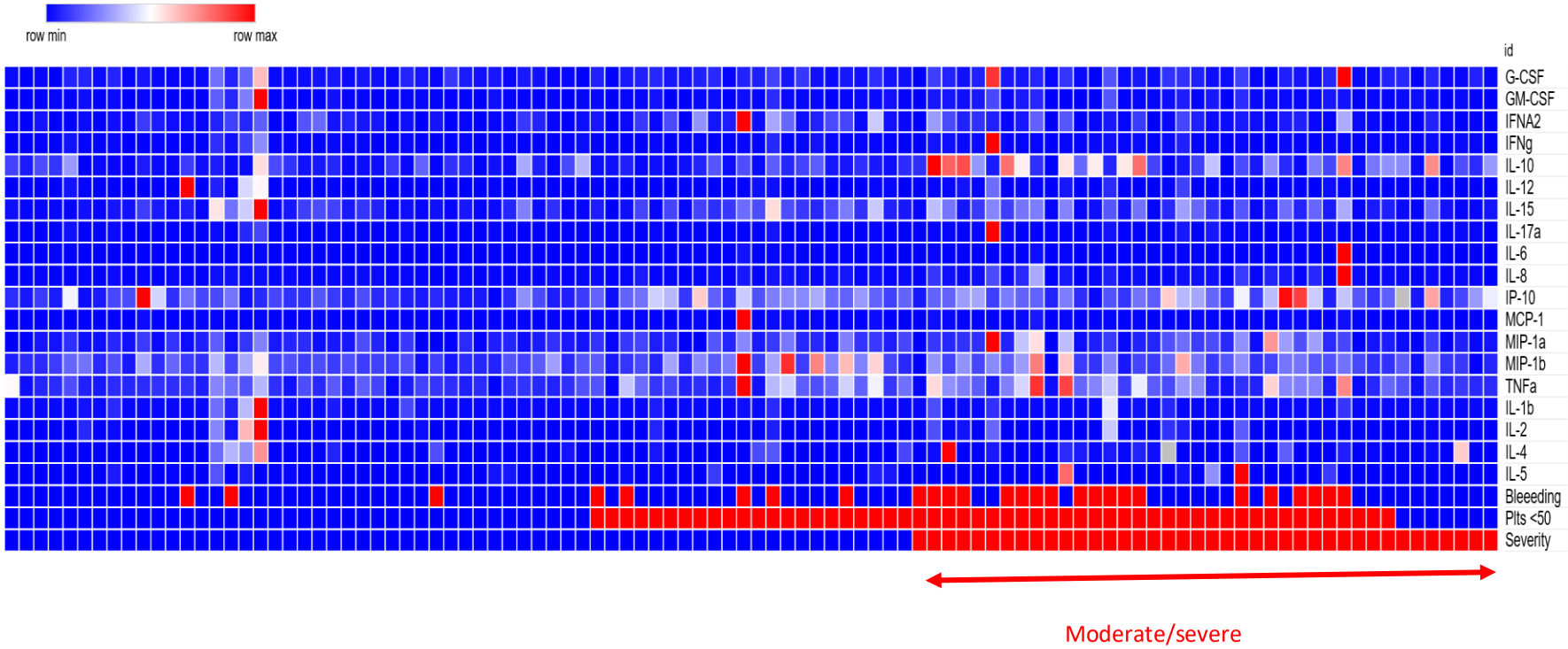


Figure 7.4 Heatmap of cytokine expression at admission by severity and lowest platelet. Dark blue signifies lowest expression, and dark red highest expression levels

7.3.4 Cytokines levels at admission by outcome

There were significant differences in distribution of cytokines at admission when groups were stratified by outcome into those that died of CCHF (fatal/survived). Higher levels of IL-10 (p=0.029), IL-15 (p=0.044), IL-6 (p=0.017), IL-8 (p=0.004), MCP-1 (p=0.01), and TNF-α (p=0.017) were observed in the fatal cases. Other cytokines including GM-CSF (p=0.078), IL-17 (p=0.072) and MIP-1α (p=0.052) were also elevated in the fatal group but not at a significant level (p<0.05).

7.3.5 Daily serial cytokines levels

Serial cytokines were measured daily in 102 participants for a median 5 days (IQR 4-7 days) during the course of acute admission (day 1-13), for a total of 580 CCHF patient days. The number of samples analysed by day of illness is shown in Table 7.5. After hospital discharge cytokine profiles were analysed in 76 of 100 eligible patients attending out-patient follow up. 60 participants attended at 14 (+2) days and 60 participants (including 46/60 from the day 14 group) at 30 (+/-2) days.

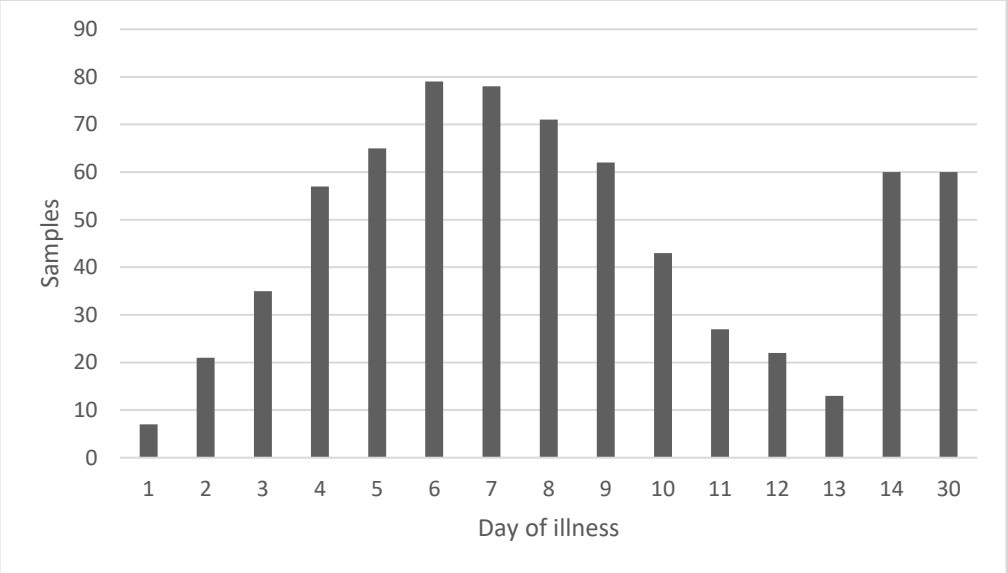
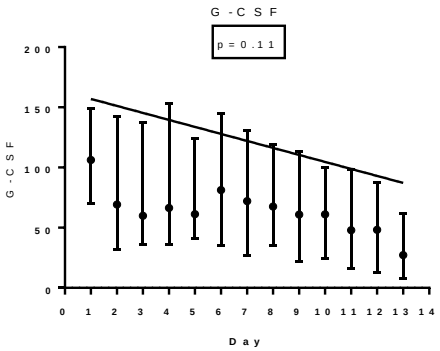
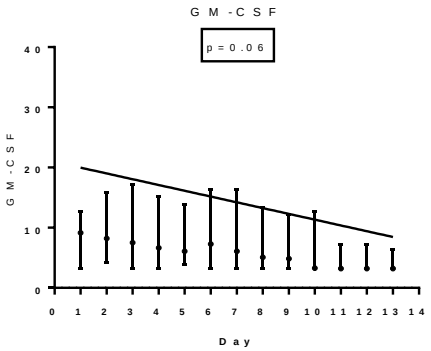


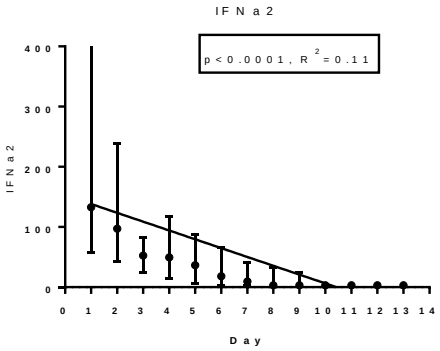
Figure 7.5 Number of different patients' samples analysed by the 19 plex cytokine panel at each day of illness.

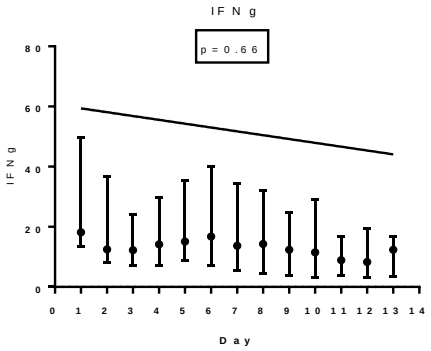
7.3.6 Linear Regression

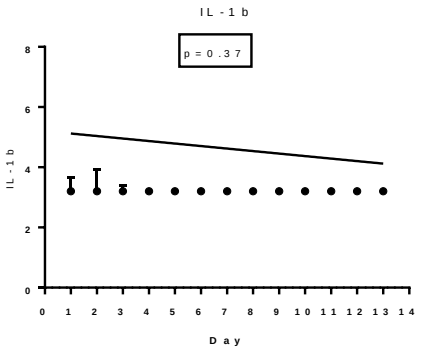
Simple linear regression between cytokine levels and day of illness is shown in Figure 7.6 for the individual cytokines. There was a significant non-zero regression slope demonstrated with a decreasing slope from onset of disease for IFN-A2 (P<0.0001), IL-5 (p=0.007), IL-10 (p=0.03), IL-15 (p<0.0001), MCP-1 (p=0.009), MIP-1α (p<0.0001), MIP-1β (p<0.0001) and TNF-α (p<0.0001).

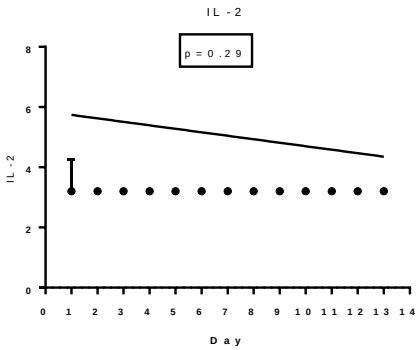

G-CSF
p = 0.11
G-CSF
Day

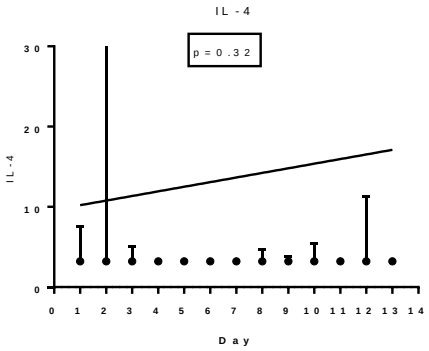

GM-CSF
p = 0.06
GM-CSF
Day

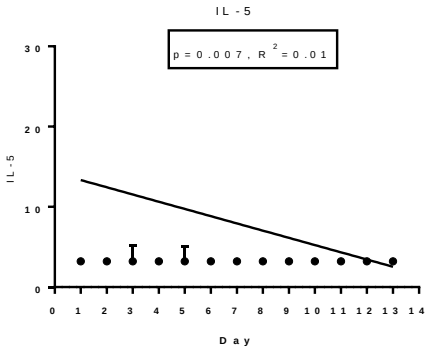

IFNa2
p < 0.0001, R 2 = 0.11
IFNa2
Day

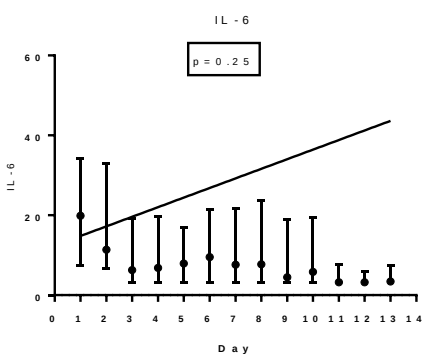

IFNg
p = 0.66
IFNg
Day

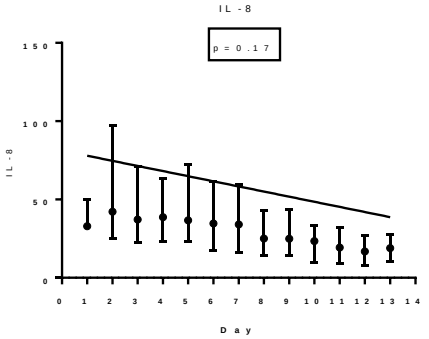

IL-1b
p = 0.37
IL-1b
Day

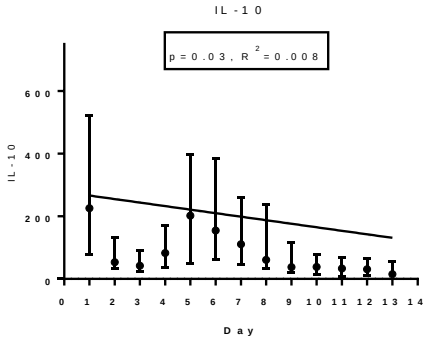

IL-2
p = 0.29
IL-2
Day

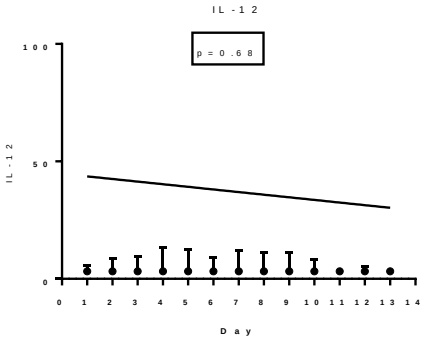

IL-4
p = 0.32
IL-4
Day

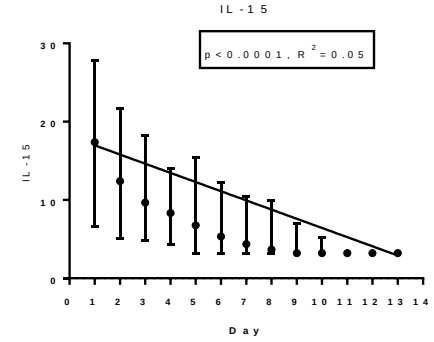

IL-5
p = 0.007, R 2 = 0.01
IL-5
Day

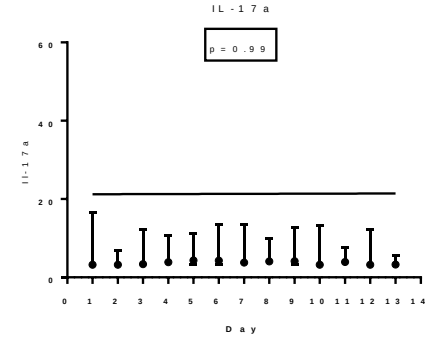

IL-6
p = 0.25
IL-6
Day

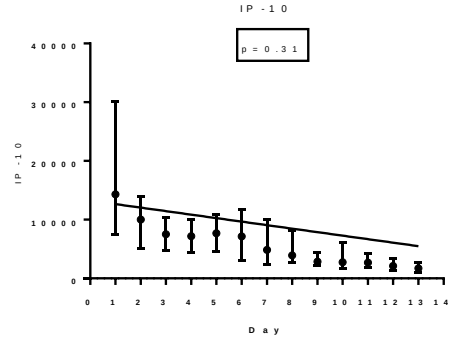

IL-8
p = 0.17
IL-8
Day

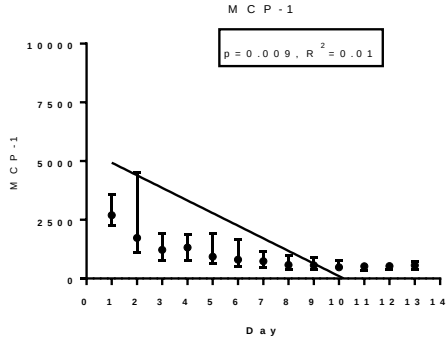

IL-10
p = 0.03, R² = 0.008
IL-10
Day


IL-12
p = 0.68
IL-12
Day


IL-15
p < 0.0001, R² = 0.05
IL-15
Day


IL-17a
p = 0.99
Il-17a
Day


IP-10
p = 0.31
IP-10
Day


MCP-1
p = 0.009, R² = 0.01
MCP-1
Day

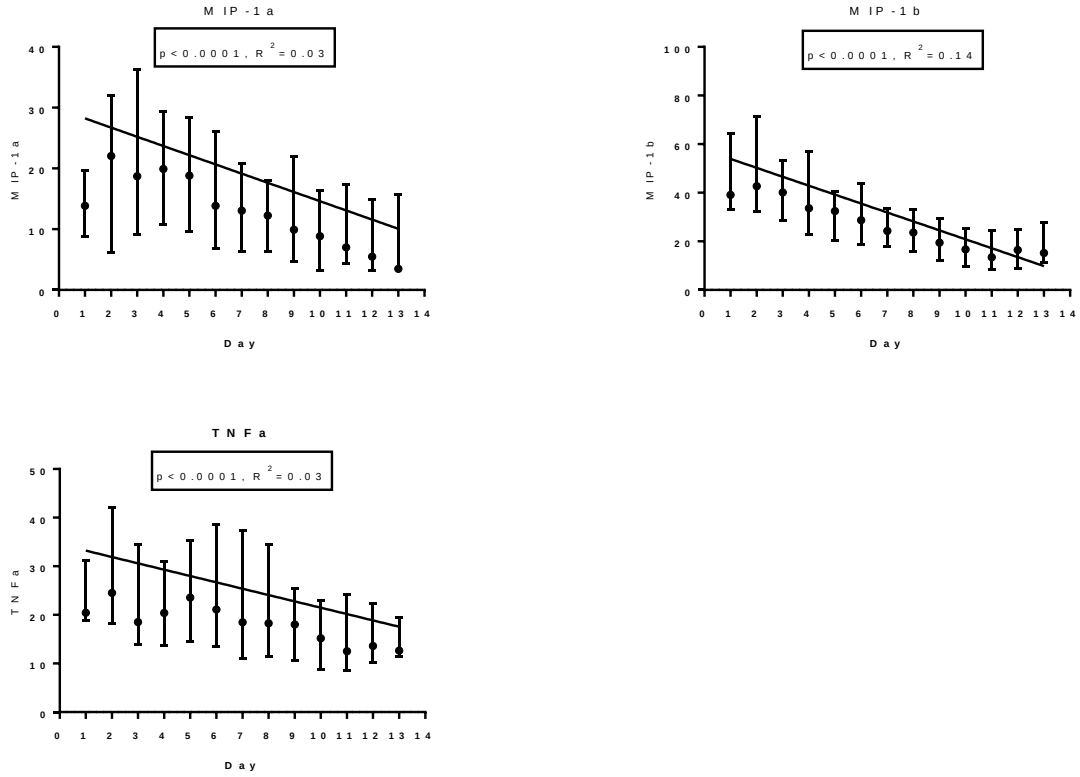


Figure 7.6 Linear regression of cytokines by day of illness. IL-5 is displayed with split y axis, to demonstrate slope and outliers (y axis pg/ml). Median and IQR are shown for each day. Il-5 is represented by an aligned dot plot.

7.3.7 Serial cytokines by severity and outcome

Linear regression analysis was then undertaken stratified by severity of illness (mild vs moderate/severe) and outcome (survived or fatal) (Figure 7.7) demonstrating 2 patterns of significant differences in cytokine expression during acute infection.

1. Cytokine slope differences

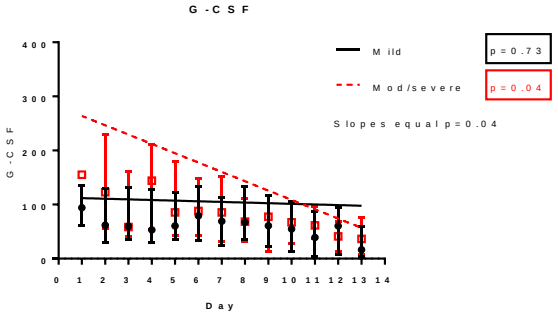
   Disease severity: G-CSF (p=0.04); IL-5 (p<0.0001); IL-8 (p=0.02), MIP-1α (p=0.001)

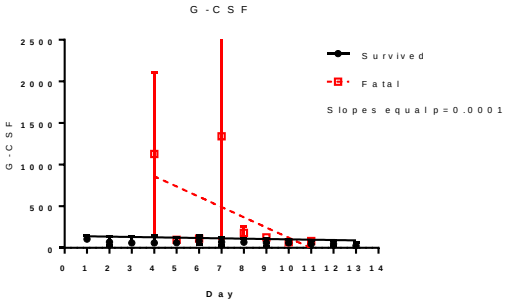
   Outcome: G-CSF (p=0.001); IL-6 (p<0.0001); IL-10 (p<0.0001); MIP-1β (p=0.043)

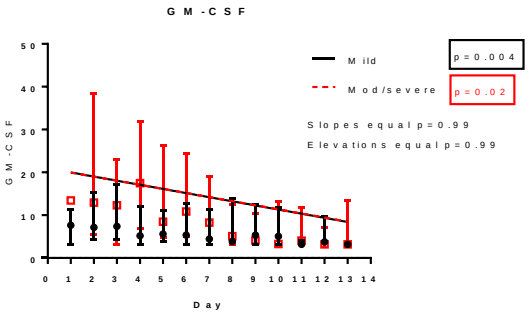
2. Higher cytokine slope elevations/intercepts

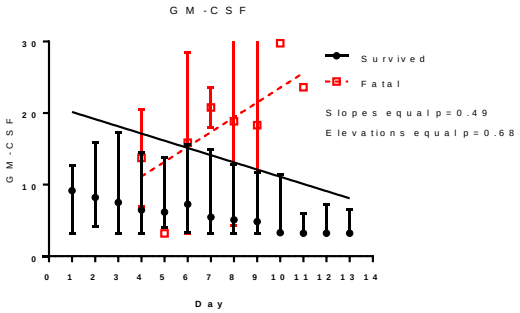
   Disease severity: IFN-γ (p=0.001); IL-10 (p<0.0001); IL-17A (p=0.004); IP-10 (p=0.029); and TNF-α (p=0.007).

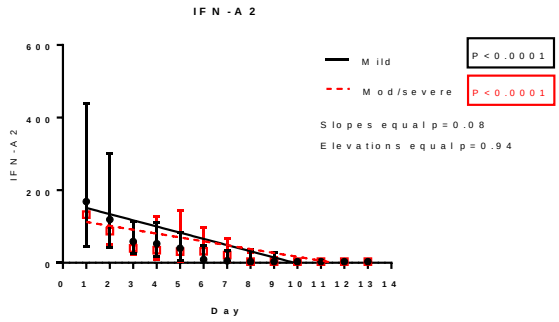
   Outcome: IL-8 (p<0.0001); IL-15 (p=0.001; and TNF-α (p=0.007)

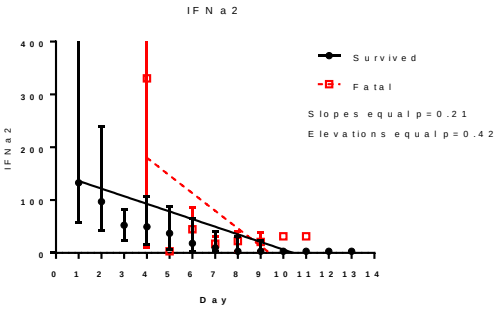

G-CSF
Mild
Mod/severe
p=0.73
p=0.04
Slopes equal p=0.04
G-CSF
Day

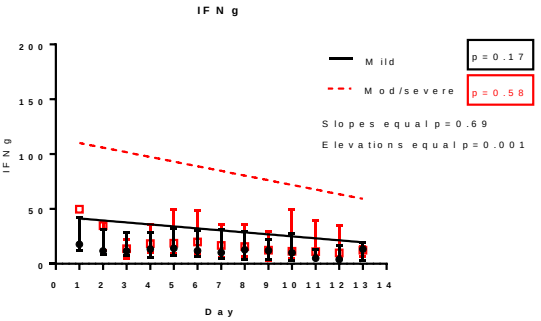

G-CSF
Survived
Fatal
Slopes equal p=0.0001
G-CSF
Day

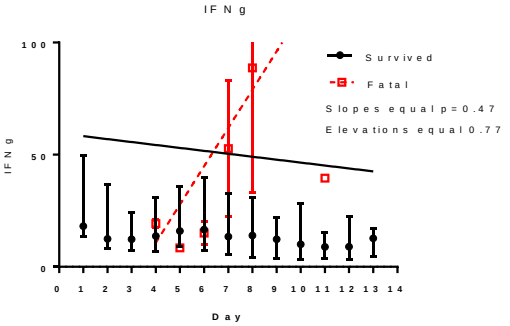

GM-CSF
Mild
Mod/severe
p=0.004
p=0.02
Slopes equal p=0.99
Elevations equal p=0.99
GM-CSF
Day

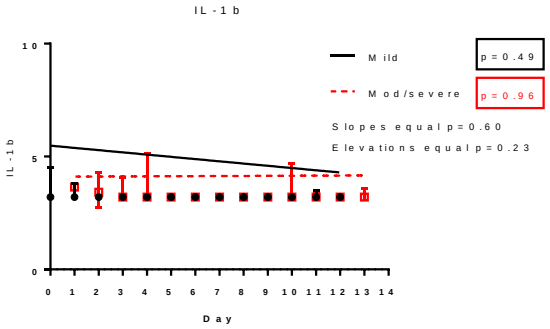

GM-CSF
Survived
Fatal
Slopes equal p=0.49
Elevations equal p=0.68
GM-CSF
Day

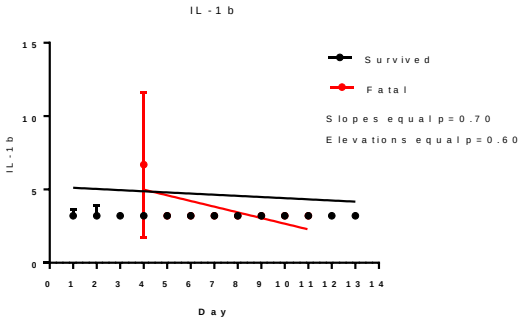

IFN-A2
Mild
Mod/severe
P<0.0001
P<0.0001
Slopes equal p=0.08
Elevations equal p=0.94
IFN-A2
Day

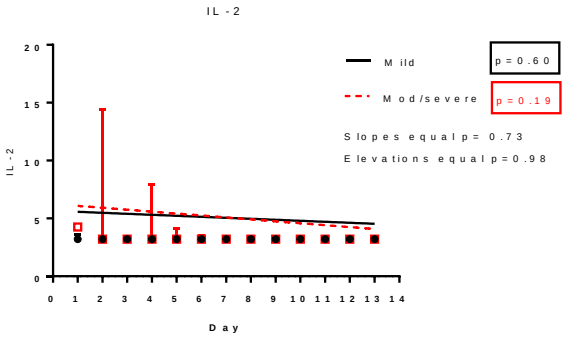

IFNa2
Survived
Fatal
Slopes equal p=0.21
Elevations equal p=0.42
IFNa2
Day

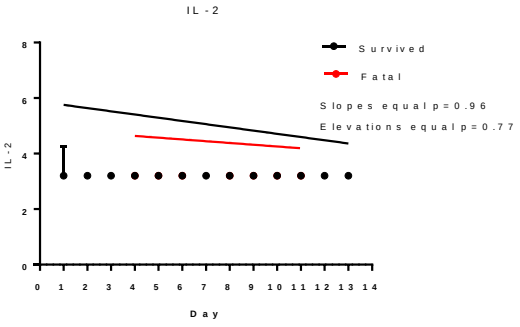

IFNg
Mild
Mod/severe
p=0.17
p=0.58
Slopes equal p=0.69
Elevations equal p=0.001
IFNg
Day

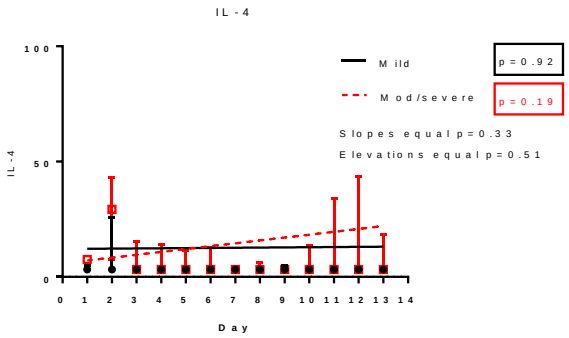

IFNg
Survived
Fatal
Slopes equal p=0.47
Elevations equal 0.77
IFNg
Day

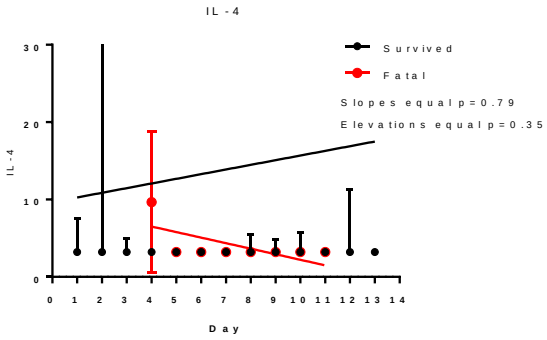

IL-1b
Mild
Mod/severe
p = 0.49
p = 0.96
Slopes equal p = 0.60
Elevations equal p = 0.23
IL-1b
Day

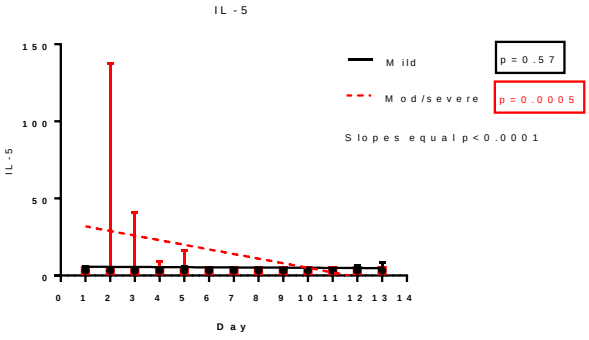

IL-1b
Survived
Fatal
Slopes equal p = 0.70
Elevations equal p = 0.60
IL-1b
Day

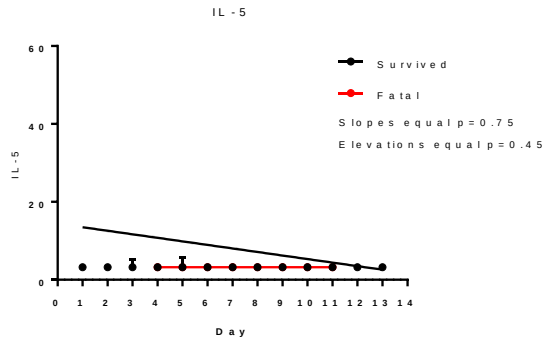

IL-2
Mild
Mod/severe
p = 0.60
p = 0.19
Slopes equal p = 0.73
Elevations equal p = 0.98
IL-2
Day

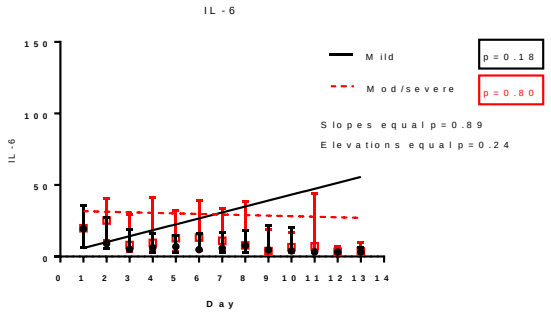

IL-2
Survived
Fatal
Slopes equal p = 0.96
Elevations equal p = 0.77
IL-2
Day

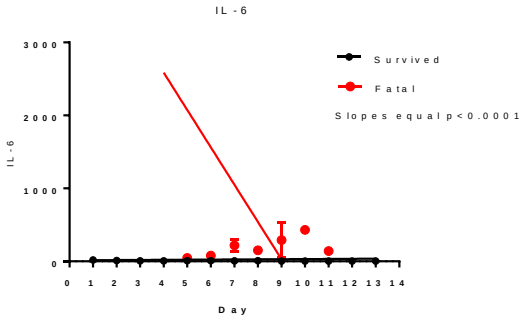

IL-4
Mild
Mod/severe
p = 0.92
p = 0.19
Slopes equal p = 0.33
Elevations equal p = 0.51
IL-4
Day

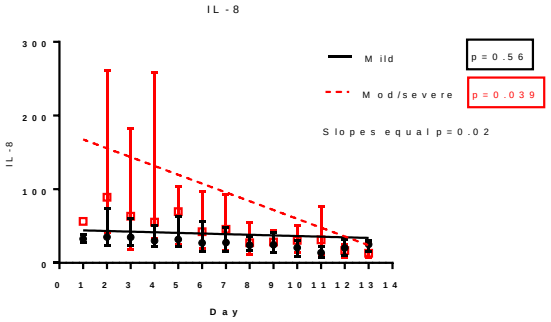

IL-4
Survived
Fatal
Slopes equal p = 0.79
Elevations equal p = 0.35
IL-4
Day

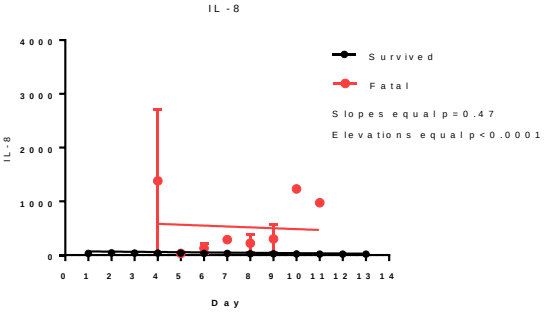

IL-5
Mild
Mod/severe
p = 0.57
p = 0.0005
Slopes equal p < 0.0001
IL-5
Day

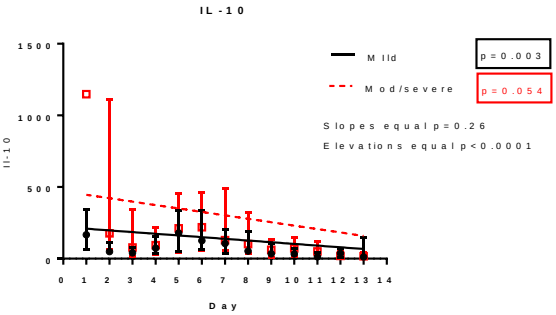

IL-5
Survived
Fatal
Slopes equal p = 0.75
Elevations equal p = 0.45
IL-5
Day

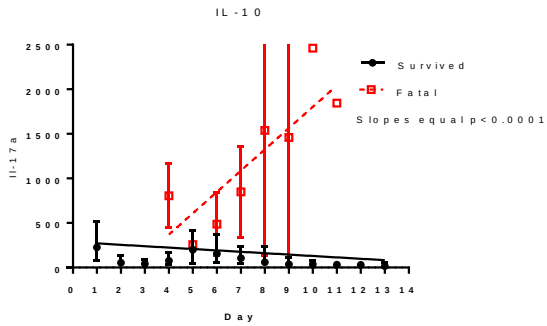

IL-6
Mild
Mod/severe
p=0.18
p=0.80
Slopes equal p=0.89
Elevations equal p=0.24
IL-6
Day

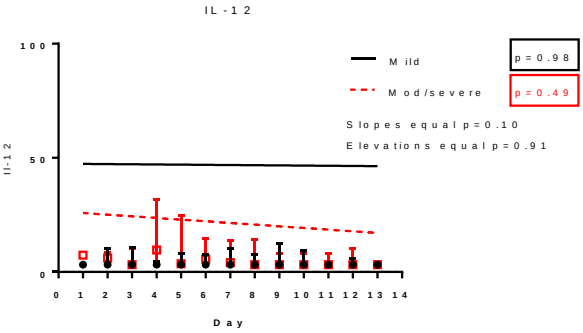

IL-6
Survived
Fatal
Slopes equal p<0.0001
IL-6
Day

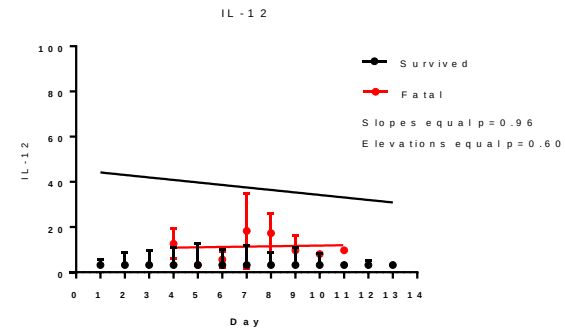

IL-8
Mild
Mod/severe
p=0.56
p=0.039
Slopes equal p=0.02
IL-8
Day

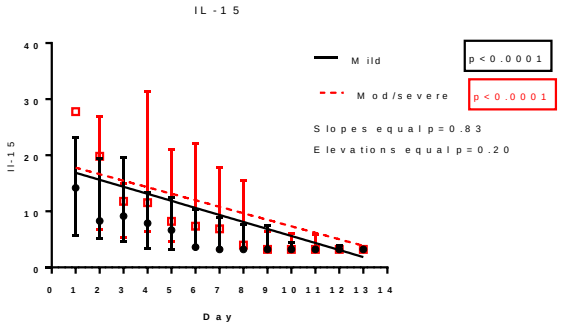

IL-8
Survived
Fatal
Slopes equal p=0.47
Elevations equal p<0.0001
IL-8
Day

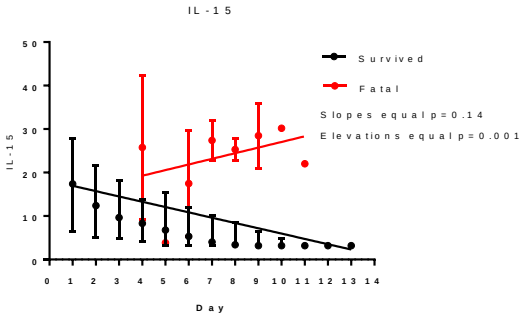

IL-10
Mild
Mod/severe
p=0.003
p=0.054
Slopes equal p=0.26
Elevations equal p<0.0001
Il-10
Day

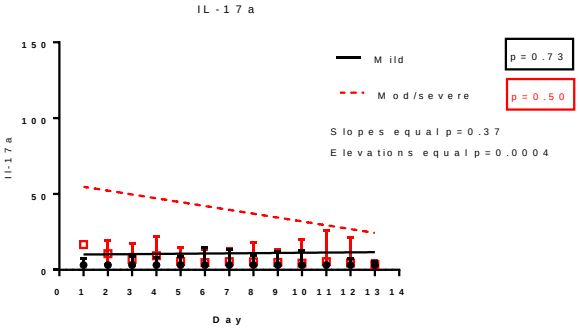

IL-10
Survived
Fatal
Slopes equal p<0.0001
Il-17a
Day

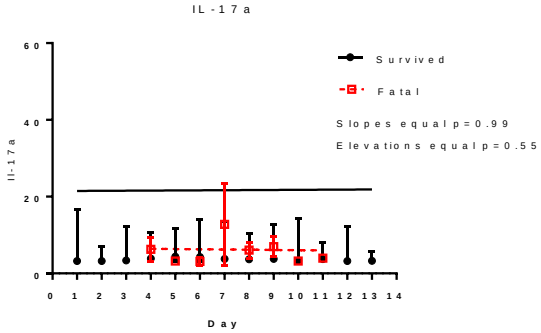

IL-12
Mild
Mod/severe
p=0.98
p=0.49
Slopes equal p=0.10
Elevations equal p=0.91
Il-12
Day

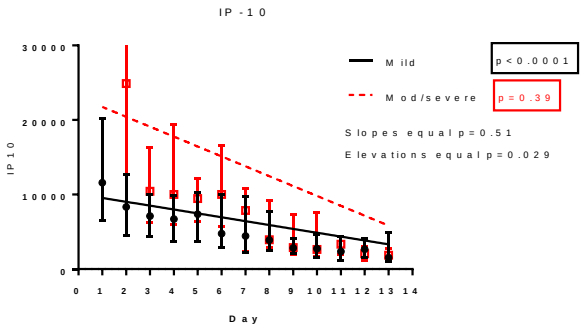

IL-12
Survived
Fatal
Slopes equal p=0.96
Elevations equal p=0.60
IL-12
Day

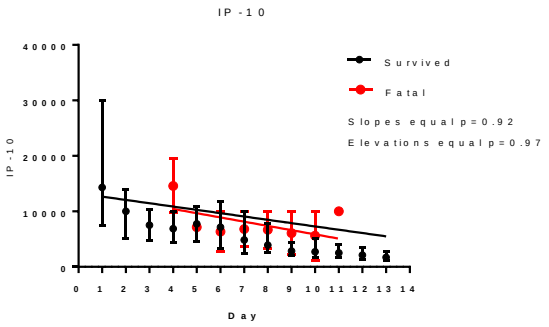

IL-15
Il-15
Mild
Mod/severe
p<0.0001
p<0.0001
Slopes equal p=0.83
Elevations equal p=0.20
Day

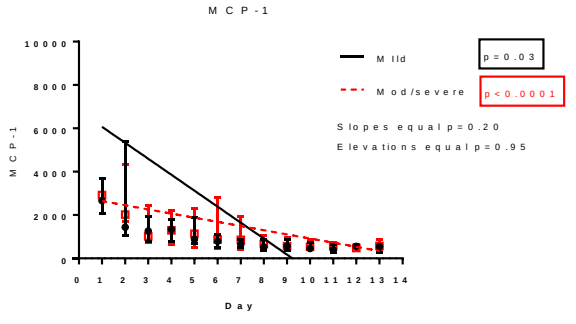

IL-15
IL-15
Survived
Fatal
Slopes equal p=0.14
Elevations equal p=0.001
Day

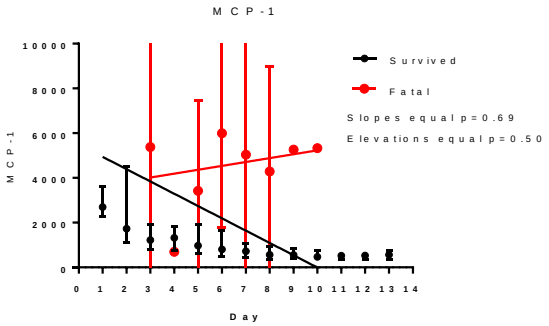

IL-17a
Il-17a
Mild
Mod/severe
p=0.73
p=0.50
Slopes equal p=0.37
Elevations equal p=0.0004
Day


IL-17a
Il-17a
Survived
Fatal
Slopes equal p=0.99
Elevations equal p=0.55
Day


IP-10
IP10
Mild
Mod/severe
p<0.0001
p=0.39
Slopes equal p=0.51
Elevations equal p=0.029
Day


IP-10
IP-10
Survived
Fatal
Slopes equal p=0.92
Elevations equal p=0.97
Day


MCP-1
MCP-1
Mild
Mod/severe
p=0.03
p<0.0001
Slopes equal p=0.20
Elevations equal p=0.95
Day


MCP-1
MCP-1
Survived
Fatal
Slopes equal p=0.69
Elevations equal p=0.50
Day

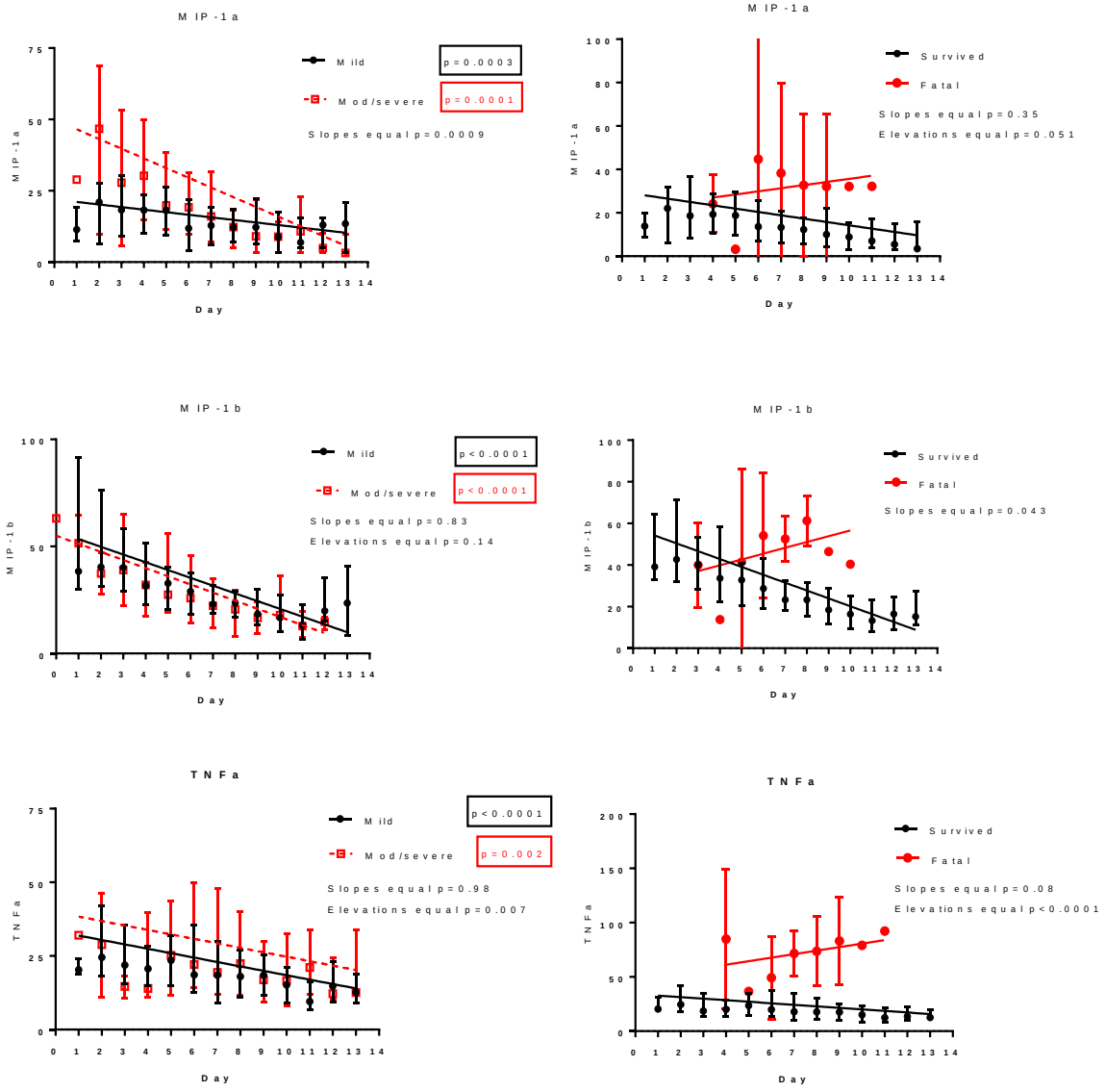
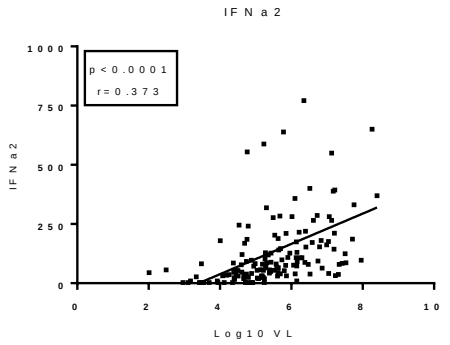
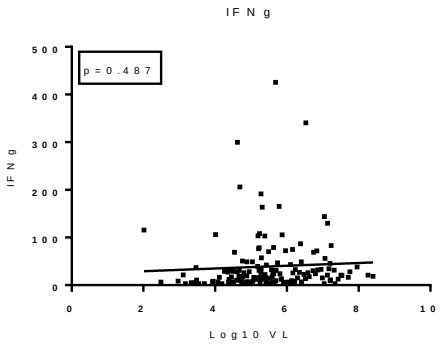


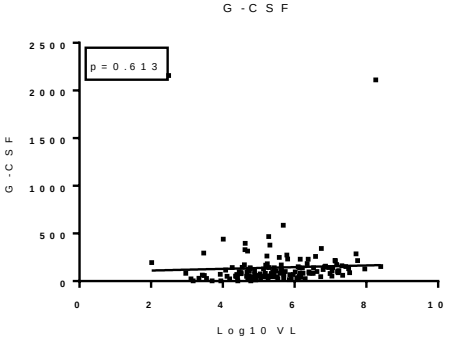
Figure 7.7 Linear regression of cytokines by day of illness stratified by disease severity and outcome (y axis pg/ml). Median (IQR) cytokine levels are shown for each day. p-values indicate if slopes are significantly non-zero, and if slopes or intercepts/elevations are equal.

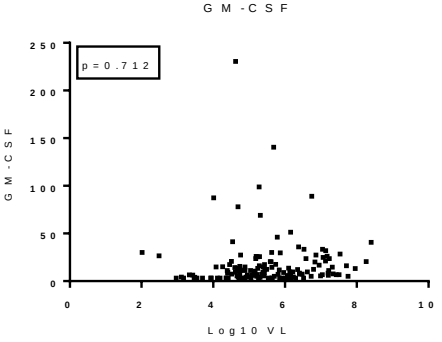
7.3.8 Cytokine level correlations with Log10 CCHF viral load

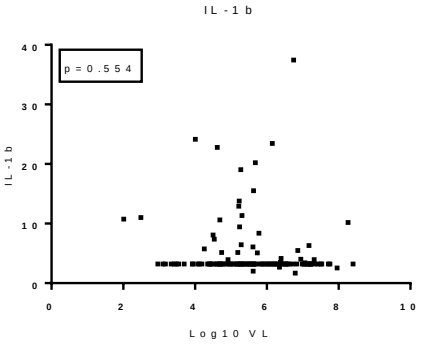
There were 149 matched daily cytokine and viral load samples, with a quantifiable CCHFV result. Significant positive correlations between individual cytokine and log10 viral load were shown with IFN-α2, IL-8, IL-10, IL-15, IP-10, MIP-1α, MIP-1β and TNF-α (Figure 7.8). The most marked correlations (r=0.3-0.5) occurred with IFN- α2, IL-8, IL15, MIP-1α, MIP-1β and TNF-α, .

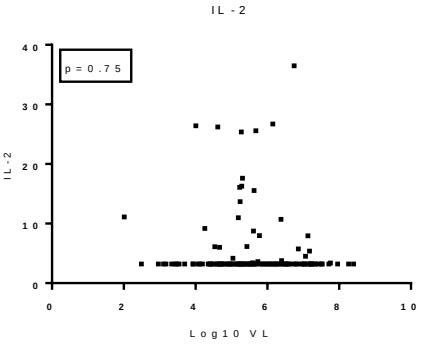

IFNa2
p < 0.0001
r = 0.373
IFNa2
Log10 VL

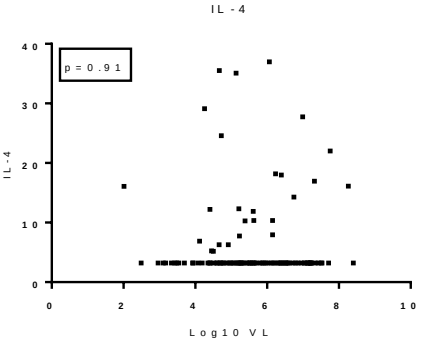

IFNg
p = 0.487
IFNg
Log10 VL

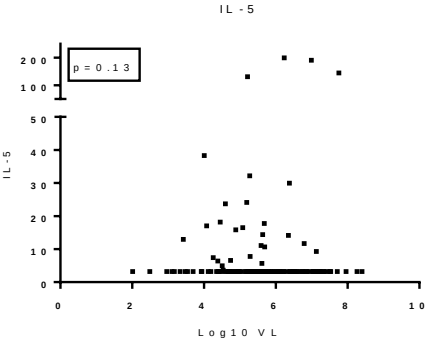

G-CSF
p = 0.613
G-CSF
Log10 VL

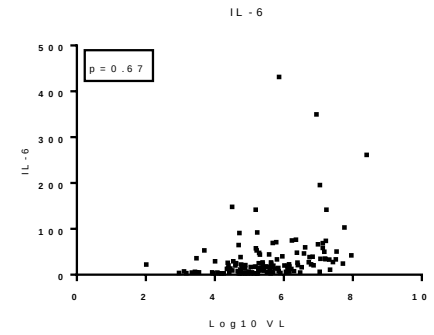

GM-CSF
p = 0.712
GM-CSF
Log10 VL

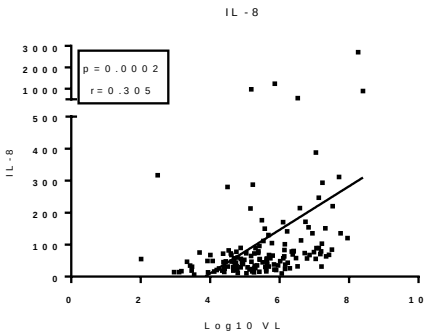

IL-1b
p = 0.554
IL-1b
Log10 VL

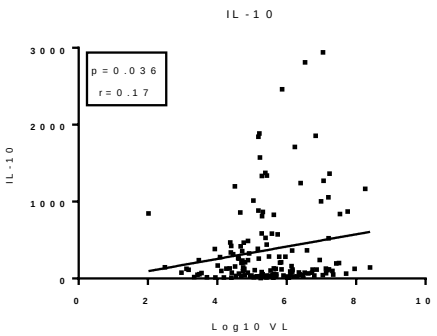

IL-2
p = 0.75
IL-2
Log10 VL

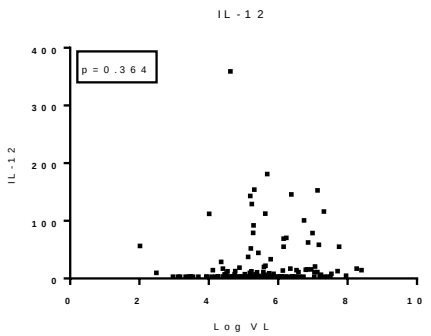

IL-4
p = 0.91
IL-4
Log10 VL

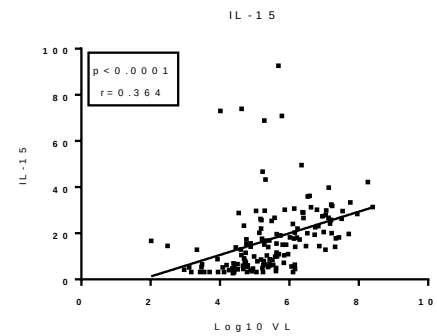

IL-5
p = 0.13
IL-5
Log10 VL

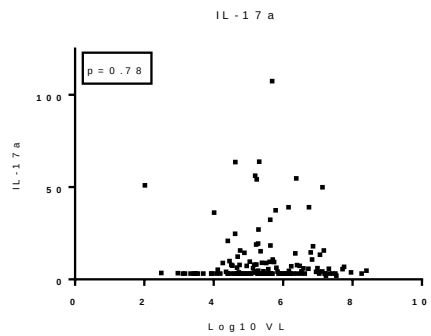

IL-6
p = 0.67
IL-6
Log10 VL

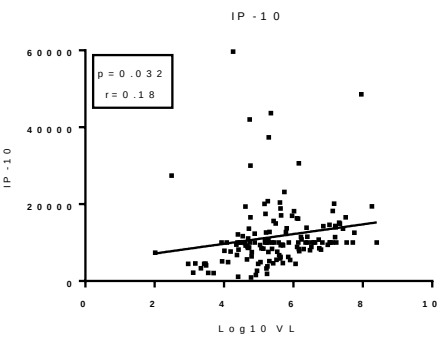

IL-8
p = 0.0002
r = 0.305
IL-8
Log10 VL

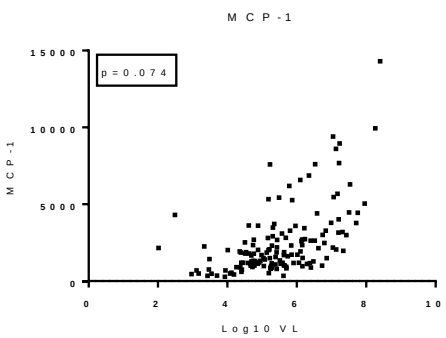

IL-10
p = 0.036
r = 0.17
IL-10
Log10 VL


IL-12
p = 0.364
IL-12
Log VL


IL-15
p < 0.0001
r = 0.364
IL-15
Log10 VL


IL-17a
p = 0.78
IL-17a
Log10 VL


IP-10
p = 0.032
r = 0.18
IP-10
Log10 VL


MCP-1
p = 0.074
MCP-1
Log10 VL

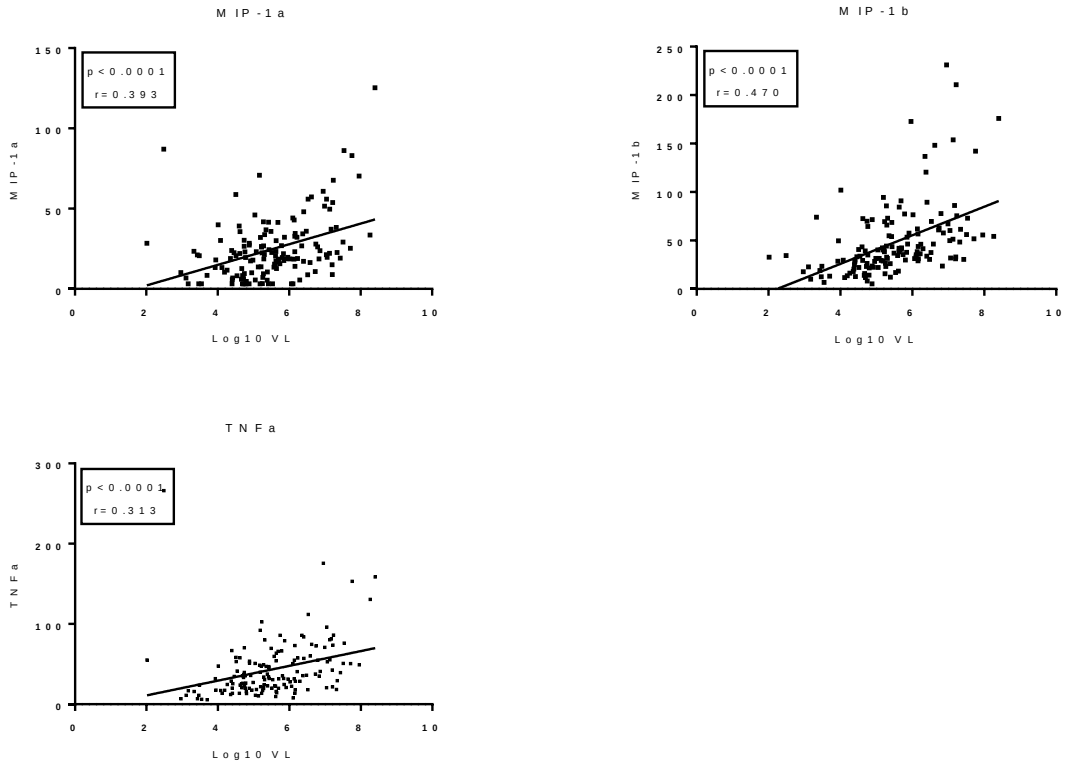


Figure 7.8 Scatter plots of cytokine levels correlated with Log10 CCHF viral load (n=149) r= Pearson's correlation coefficient. Best fit lines included in significant ($p<0.05$) results (y axis pg/ml, x axis Log10 copies/ml).

Cytokine	Admission vs convalescence p-value	Admission by severity p-value	Admission by Plt count p-value	Admission by outcome p-value	Admission correlation Viral load p-value	Linear regression slopes p-value
G-CSF	**<0.0001**	0.54	**0.02**	0.23	0.33	0.14
GM-CSF	**0.002**	**0.024**	**0.01**	0.078	0.53	0.06
IFN-A2	**<0.0001**	0.55	**0.01**	0.15	**<0.0001**	**<0.0001**
IFN-γ	**<0.0001**	0.044	0.13	0.62	0.33	0.73
IL-10	**<0.0001**	**<0.0001**	**0.02**	**0.029**	0.259	**0.03**
IL-12	**<0.0001**	**0.022**	0.137	0.17	0.19	0.68
IL-15	**<0.0001**	0.38	**0.001**	**0.044**	**<0.0001**	**<0.001**
IL-17A	0.90	**0.013**	0.39	0.072	0.26	0.99
IL-1β	0.42	0.88	0.57	0.43	0.69	0.37
IL-2	0.87	0.11	0.39	0.59	0.36	0.29
IL-4	**0.004**	0.031	0.20	0.63	0.64	0.32
IL-5	0.39	0.31	0.59	0.67	0.87	**0.007**
IL-6	**<0.0001**	0.11	**0.0001**	**0.017**	**0.03**	0.25
IL-8	**<0.0001**	**0.009**	**<0.0001**	**0.004**	**0.0002**	0.17
IP-10	**<0.0001**	**0.015**	**<0.0001**	0.28	0.85	0.31
MCP-1	**<0.0001**	0.66	**0.001**	**0.01**	0.074	**0.009**
MIP-1α	**0.0004**	0.088	**0.001**	0.052	**0.051**	**<0.0001**
MIP-1β	**<0.0001**	0.81	**0.006**	0.20	**<0.0001**	**<0.0001**
TNF-α,	**<0.0001**	0.067	**0.002**	**0.017**	**<0.0001**	**<0.0001**

Table 7.5 Summary of cytokine results

7.4 Discussion

Cytokines are key proteins involved in the host immune response, and through cell signalling mechanism have both pro-inflammatory and immunomodulatory roles. Regulation of the cytokine network maintains the equilibrium of adequate detection and response to pathogens whilst limiting excessive tissue damage. Sepsis and related organ dysfunction are frequently attributed to excessive release of pro-inflammatory cytokine with a resultant 'cytokine storm'. Immunomodulation of the host immune response in sepsis has to date not been shown to be beneficial in sepsis clinical trials, but results may be the heterogenous nature of the sepsis syndrome.

This study aimed to evaluate the dynamic cytokine response in patients with CCHF longitudinally during their acute illness. This involved intensive daily sampling and analysis utilising a comprehensive 19 plex human cytokine panel that resulted in 580 cytokine results for each of the cytokines, in 102 participants with confirmed CCHF. Samples were also rapidly processed and frozen to prevent degradation, with no freeze thaw cycles and were measured in duplicate.

Baseline or admission cytokine levels in CCHF have been the focus of the majority of studies reported, often stratifying patient groups by severity or outcome. They provide useful preliminary information with potential prognostic benefit, but do not reveal the longitudinal trend of cytokine expression in acute illness and hence a deeper understanding of this component of the immune response. This research also includes a previously unstudied 'mild' severity CCHF cohort, and data on 30-day cytokine levels that should provide valuable comparator arms if deregulated cytokines are associated with CCHF infection, severe and fatal disease.

The first evaluation of the cytokines compared the 19 plex panel values at admission against values obtained in convalescence at 30 days post onset of symptoms. As might be expected from the panel selected, the levels were elevated at admission in the vast majority (15/19) of the cytokines, generally with highly statistically significant differences. The four cytokines that had no difference were IL-1β, IL-2, IL-5 and IL-17A that are all characterised as predominantly pro-inflammatory, with levels remaining low and at the limit of detection. No difference was also noted in these cytokines at admission when stratified by disease severity or outcome, suggesting that they are not playing a key role in CCHF pathogenesis.

Previous studies in CCHF that have demonstrated positive results for IL-2 and IL-5, include Ozsurekci et al [126] who showed levels to be higher in fatal cases in a small study of 33 survivors vs 3 fatal cases; there were no differences compared to controls. Parlak et al [122] conversely

found IL-2 levels to be higher in patients than controls, but no difference by disease outcome. IL-1B might be expected to demonstrate more significant results as an important pro-inflammatory cytokine and it was hypothesised to be associated with severe disease. Papa et al [123] found it to be raised in fatal cases and previous studies in animal and human bacterial sepsis suggest it is a powerful pro-inflammatory cytokine released predominantly from activated macrophages with a pyrogenic effect. IL-17A is a pro-inflammatory cytokine produced by Th2 cells that may have more of important role in inducing a pro-inflammatory response to extracellular pathogens. It has previously been investigated in autoimmune disease, inflammatory bowel disease and psoriasis [230,231]. Our results are consistent with a previous evaluation in CCHF when no difference in levels were found in adults or children compared to controls[126], although it was elevated in severe cases in our study.

The elevated levels of the remaining 15 cytokines/chemokines analysed, compared to convalescence levels suggest a role in the complex immune response to CCHFV infection. The potential to assess cytokine profiles stratified by markers of disease severity is also important. The low case fatality rates in Turkey makes it difficult to adequately compare cytokine profiles between fatal and survivor cases. Whilst acknowledging the limitations of existing scoring systems, cytokine levels were evaluated in severity groups and the pragmatic Turkish grouping of patients with a platelet count $<50 \times 10^9$/L.

There was a difference in cytokine levels at admission between disease severity/platelet count groups (Table 7.3). Our demonstration of higher levels of cytokines IL-10, IP-10 in severe vs non-severe cases at the $p<0.025$ level is consistent with previous studies. However, for the first time GM-CSF, IL-12 and IL-8 have been shown to be higher in moderate/severe cases and when severity is gauged by platelet count, MIP-1α, MIP-1β, MCP-1, Il-6, IL-15, TNF-α, INFA2 and G-CSF were also elevated in patients who have platelet counts $<50 \times 10^9$/L. Many of the previous small cytokine studies focussed on the evaluation of small panels at admission in surviving or fatal cases. The most consistently elevated cytokines in fatal cases in previous studies (n=4-5) are IL-10, IL-6 and TNF-α (Table 1.5). Even though this cohort had a limited number of CCHF deaths we also found significant associations between fatality and elevations in IL-10, IL-6 and TNF-α. We also found significant associations with mortality and higher levels of IL-15, IL-8 and MCP-1.

Intensive longitudinal analysis of large cytokine profiles during sepsis is rarely undertaken and no large comparative data sets exist. Previously in CCHF sampling has been intermittent during acute infection and limited panels of cytokines evaluated. This data demonstrates a linear relationship for a number of pro-inflammatory cytokines including IFN-A2, IL-15, MCP-1, MIP-1α, MIP-1β and TNF-α. However, all linear regression slopes were accompanied by low R-

squared values highlighting that whilst a relationship/trend exists there is significant data variability around regression lines. This is expected with regression analysis of noisy, high variability cytokine data, in a cohort with a broad spectrum of clinical illness and other contributing predictor variables that will affect response. Analysis of longitudinal cytokine responses by disease severity and outcome highlights exaggerated and persistent pro-inflammatory immune responses during infection associated with severe disease and death. This is demonstrated through significantly different slopes or slope elevations in G-CSF, IL-6, IL-8, IL-15, MCP-1, MIP-1α, MIP-1β and TNF-α. IL-10 is a key anti-inflammatory cytokine, that also showed different trends during illness course with elevated levels in moderate/severe disease, and increasing levels during illness with a fatal outcome.

Looking at all the individual cytokines, there are clear patterns of a relationship between certain cytokines and severity, independent of the method utilised for stratifying groups for comparison. These include IL-10, IL-15, IL-6, IL-8, IP-10, MIP-1α and TNF-α (Figure 7.4). TNF-α is one of the most studied pro-inflammatory cytokines in VHF and sepsis more generally, and is sometimes referred to as the master regulator of inflammatory cytokine production [232]. It has also been implicated in a number of non-infectious diseases such as rheumatoid arthritis [233] and atherosclerosis [234]. It is mainly derived from macrophages shortly after they are activated and after release acts via specific receptors, activating other immune cells and release of other immunoregulatory mediators (IL-6,IL-8) [113]. The ratio between TNF-α and its receptors, has also been shown to be important in patients with sepsis [235], whilst injection of TNF-α into animals and humans [236–238] results in a sepsis-like syndrome also generating fever. It has many of its direct effects on macrophages, enhancing their production, activation, differentiation and survival that further promotes a pro-inflammatory response [113]. TNF-α also induces expression of tissue factor that can initiate the coagulation cascade [196]. In this study TNF-α levels were higher in acute samples, more severe disease and in fatal cases. Longitudinal analysis showed that survivors had different expression patterns to fatal cases, with a linear reduction in TNF-α during illness course in survivors. By disease severity the slopes were similar but elevated in the moderate/severe group.

IL-6 is another important pro-inflammatory cytokine that has a broad range of immune effects as a including as a mediator of the acute phase response [239,240], and a role in the activation of B and T lymphocytes. Unlike TNF-α, the injection of IL-6 has not been shown to result in a sepsis clinical picture in animals [241], but has been implicated in causing myocardial depression in meningococcal disease [242]. It also has important anti-inflammatory responses, including inhibition of TNF-α and the release and increased production of IL-10 [243]. Whilst IL-6 has been shown to be elevated in acute, more severe and fatal cases in this study there was no clear

linear trend identified. This may support its mixed anti and pro-inflammatory response in contrast to the heightened pro-inflammatory response observed in TNF-α earlier in disease course.

IL-15 is increasingly recognised as an important cytokine in sepsis and septic shock, due to its role maintaining the homeostasis of natural killer (NK) and memory CD8 T lymphocytes [244], that have been shown to promote the pathophysiology of sepsis [245,246]. IL-15 has been shown to result in increased production of IFN-γ, and also prevent apoptosis through up-regulation of anti-apoptotic proteins [247]. IL-15 knockout mice show resistance against sepsis [244,248] and elevated levels of IL-15 have been demonstrated in patients with severe sepsis [249,250]. In a large cohort of patients with Stevens-Johnson syndrome IL-15 (and IL-6, IL-8 and TNF-α) was upregulated, and was the only factor to correlate with disease severity and mortality [251]: IL-15 has also been shown to correlate with invasive fungal infection in battlefield trauma patients [252]. There is ample evidence to indicate that the innate immune system including NK cells are also activated early during the course of primary and secondary DENV infection. The ability of NK cells to secrete cytolytic granules has long been recognized, and they are considered a crucial first line of defence in eliminating DENV-infected cells (221). Flow cytometry has also shown that the frequencies of activated NK cells are higher in patients with DHF compared to those with DF [253].

In this study IL-15 has been shown to be significantly elevated in acute infection, fatal cases and those who developed low platelet count counts. It also showed a clear correlation with CCHF viral load and markedly reduced during the course of acute illness/infection. The fatal cases had evidence of septic shock that would be consistent with previous reports of elevated IL-15, but the other mild and moderate/severe cases did not develop severe sepsis/septic shock, consistent with the lack of difference in IL-15 levels between disease severity groups. Yilmaz et al [254] reported that there more peripheral blood NK cells in severe-risk CCHF patients than in non-severe risk group CCHF patients, and found a positive correlation between NK cell count and aspartate transferase (AST), alanine transferase (ALT) and activated partial thromboplastin times (aPTT). Another report by Akinci et al [110] did not demonstrate any correlation with NK cells and fatal disease. It is clear from these data that IL-15 plays a dynamic role in the host immune response to CCHFV aligned with its pro-inflammatory response in maintaining NK cell integrity and function. There is an early heightened IL-15 response in acute infection, that fails to reduce and is increased in fatal disease.

A number of pro-inflammatory chemokines demonstrated elevated levels in more severe and fatal disease, with clear trends and correlations with CCHF viral load observed for the first time.

The three with the most consistent statistically significant elevated results are IP-10 (CXCL10), IL-8 (CXCL8) and MIP-1α. Chemokine signalling elicited from infected cells contributes to both innate and adaptive immune responses that control growth of the invading pathogen [255]. Elevated IP-10 levels have been shown to occur in broad range of infections, and in particular has been shown to have a strong correlation with HIV viral load [256], and to be elevated in children with sepsis [257] and patients with severe malaria and multi-organ dysfunction [258]. IP-10 is a potent pro-inflammatory chemokine that chemoattracts CXCR3-positive cells, including macrophages, dendritic cells, NK cells and activated T lymphocytes (CD4+ Th cells, CD8+ Tc cells) towards infected sites. Its powerful chemotactic action on activated lymphocytes allows it to modulate both innate and adaptive immunity, whilst also having an important role inducing apoptosis [259]. In 2 small previous retrospective studies (total 55 patients) by Papa et al [107,123], IP-10 was elevated in patients compared to controls and also in severe/fatal cases. In this study IP-10 levels were elevated in acute infection and severe disease, and correlated with CCHF viral load during hospitalisation. They were not associated with fatal outcome, and the ongoing expression during acute infection may indicate its role in the adaptive immune response.

IL-8 (CXCL8) is a pro-inflammatory chemokine that is produced by macrophages and endothelial cells and predominantly promotes chemotaxis and degranulation of neutrophils. It has been suggested as an early biomarker for the diagnosis of neonatal sepsis and sepsis occurring in burns patients (228,229). It has also been evaluated in one previous CCHF study in Turkey, that showed it was higher in cases versus controls and in fatal cases [124]. Our data showed that IL-8 was elevated in acute infection, more severe disease, fatal outcome and also significantly correlated with CCHF viral load. There was no clear trend of expression during acute infection by linear regression, consistent with the previous limited evaluation in CCHF. The endothelium plays a pivotal role in the host response in sepsis and these elevations provide further evidence of endothelial cell activation in CCHF. MIP-1α/CCL3 (Macrocyte inflammatory protein alpha /chemokine ligand 3) is produced predominantly by macrophages and acts as a pro-inflammatory cytokine, and with related CCH chemokines can mobilise a range of monocyte-lineage cells and lymphocytes into inflammatory tissue. It can also induce synthesis of IL-5, IL-6 and TNF-α, and has been shown with CCL4 (MIP1β), and CCL5 (RANTES) to inhibit the release of the pro-inflammatory cytokine IL-1β [260]. We have demonstrated that elevated MIP-1α levels are associated with acute infection, outcome and viral load, with clear and marked reduced levels during the course of symptomatic infection. Similar trends were also observed for MIP-1β that also correlated with acute infection, low platelet counts and CCHF viral load.

The opportunity to evaluate cytokine profiles with matched CCHF viral load data is rarely available, but has been done on for limited cytokine panels previously by Saksida et al [97] (n=46

patients at admission, IL-10, IL-12, IFN-γ, TNF-α), Papa et al [107](n= 35 patients at admission, IP-10) and Kaya et al [98] (n=31 sequential samples, Il-6, TNF-α, , IL-10 and IFN-γ). As shown in Table 1.5 correlations between CCHF viral load and cytokine levels have been demonstrated twice previously for TNF-α, , once for Il-6 and IP-10, with mixed results for IL-10 and IFN-γ. At admission we also demonstrated correlations between viral load and a range of cytokines/chemokines including TNF-α and IL-6, but not for IP-10, IL-10 or IFN-γ. However, when the data was expanded to evaluate the cytokine correlations in all CCHFV positive longitudinal samples IL-10 and IP-10 positive correlations were demonstrated. As we and others have already demonstrated there are significant associations between disease severity and CCHFV viral load. There is a clear relationship between the quantitative levels of both CCHFV blood and the expression of a range of cytokines and chemokines, that are contributing to eliminate virus and limit tissue damage.

IL-12 is a pro-inflammatory cytokine that we had hypothesised would be reduced in severe disease. Its role in sepsis remains controversial [113], with lower levels in fatal cases of severe sepsis [261,262]. It is produced predominantly by innate immune cells, including epithelial cells, dendritic cells, and macrophages. It plays a critical role in inducing Th1 responses [263] with enhanced mononuclear phagocyte responses [113], leading to the production of a number of cytotoxic cytokines, as well as interferon-gamma. In the study levels were significantly higher in acute infection compared to convalescence and in severe disease, but showed no clear trend on longitudinal sampling.

IL-10 is a key anti-inflammatory cytokine produced by Th2 cells, monocytes and B-cells that inhibits the expression of TH1 cytokines. It has been shown be elevated in levels of in patients with severe sepsis, sepsis scores and fatal outcome [264,265]. Previous research in CCHF has demonstrated mixed results but higher levels have been predominantly associated with fatal outcome [119,122–124], apart from in one study [120]. Our data clearly shows that it is an important cytokine in the host immune response to CCHFV infection. It was elevated in acute infection compared to convalescence and by all markers of increased severity and outcome. Its expression during the course of illness also demonstrated significantly different slopes in fatal and surviving cases.

The cytokine profiles that we have identified in patients with CCHF represent a valuable tool for the characterisation of immunological response patterns. In the future they may also be combined with other prognostic indicators such as viral to aid identification of patient groups at risk for developing severe disease. However, whilst trying to make a direct link between cytokine concentrations and the severity of CCHF is appealing, the spectrum of CCHF severity

cannot solely be attributed to the actions of single or small combinations of cytokines. Our results may be highlighting a complex relationship between a range of cytokines, including some not analysed and other immunological and virological factors involved. Overall, the longitudinal serial measurement of cytokines/chemokines indicates a strong pro-inflammatory response early in the disease course, potentially focussed on activation and chemoattraction of NK cells, B cells and the stimulation of acute phase proteins. TNF-α is clearly an important cytokine and in combination with other pro-inflammatory cytokines remains elevated/dysregulated in fatal disease. Patterns of IL-10 expression are similar, demonstrating that the immune response to CCHF is not a simple model and that both pro- and anti-inflammatory responses occur simultaneously, that may act to both eliminate CCHFV and limit excessive collateral tissue damage.

This data has for the first time also shown the expression of different cytokines during the course of illness and clearance of CCHF, as well the correlations with CCHF viral load. Whilst the priority for future clinical research in CCHF should focus on evaluating and antiviral/immune based therapeutics, there may also be role in investigating opportunities to target specific steps of the interactive and dynamic process of the cytokine-based host immune response.

Chapter 8 Discussion

CCHF is a major emerging infectious threat and although it is widely distributed and increasingly reported, the pathogenesis of disease is poorly understood. This is mainly due to the current necessity to perform research in advanced biosafety laboratories and the occurrence of infections in areas where research facilities are limited. Until recently there has also not been an established animal model, and the first reports of CCHFV disease in a non-human primate model need confirmation [266]. The role of ribavirin antiviral therapy continues to be controversial, but an improved understanding of disease pathogenesis should inform new approaches to improving clinical outcomes.

The specific aims of this research were to recruit a cohort of adults with PCR positive CCHF in Turkey to:

1. Determine the viral load dynamics in serum and urine over the course of clinical infection.
2. Investigate the hypothesis that: (1) initial viral load (day 1) and (2) rate of viral load reduction are correlated with disease severity and clinical outcome.
3. Investigate the host immune response to clinical infection through serial measurement of cytokine/chemokine levels and quantitative serological response, related to disease severity and clinical outcomes.
4. Test the hypothesis that severe/fatal CCHF is associated with a deregulated cytokine network characterised by an exaggerated pro-inflammatory cytokine response (IL-1β, IL-6, IL-8 and tumour necrosis factor alpha [TNF-αl], and reduced IL-12).

In this study, three research sites and two laboratories were established in the CCHF hyperendemic region and one hundred and forty-four participants with suspected CCHF were recruited, of which one hundred and four were found to be CCHFV PCR positive. The demographics of the cohort were similar to larger published epidemiological studies in Turkey, although the median age was higher than was expected (50 years), but with a low frequency identified comorbidities. The majority of patients had a history of tick bite (70%), consistent with data across Turkey and presented rapidly to hospital, a median of 3 days after symptom onset. A high percentage had close animal contact (82%) with 50% having direct contact with animal blood or tissue. Tick bite remains the main transmission risk factor for CCHF, whilst in other CCHF endemic countries direct contact with animal blood and tissue may be more frequent. Differences in the mode of transmission between different endemic countries influence control measures, but may also have implications with respect to viral inoculum dose and the severity of clinical disease encountered.

8.1 Clinical and laboratory features

The clinical features of the participants at admission is consistent with larger data sets, although bleeding was less frequent. This probably reflects recruitment of patients directly from Tokat State hospital, that as secondary level healthcare facility manages higher rates of early/mild disease, in contrast to the tertiary CCHF treatment centres where most research has been undertaken. There are limited data on vital signs at admission in patients with CCHF; in this study the vast majority were normal, with median NEWS scores of 2 and 1 in moderate/severe and mild disease groups respectively. A history of fever was reported by 90% of patients with CCHF with the mean temperature at admission 37.5^0C. This has important implications for case definition for surveillance and screening and is consistent with data on Lassa fever from Nigeria [94] and from the Ebola virus disease outbreak in West Africa [181], that 'high fever' is not always present.

The ability to predict mortality in patients with CCHF, through utilisation of severity scoring systems would be extremely useful. This is especially relevant in endemic settings when managing large cohorts, but scoring systems are not in routine use in most specialist CCHF centres in Turkey. Whilst the SSI and SGS systems have been validated in cohorts in Turkey for this aim, improvement in their predictive value is required for other key outcomes such as blood product requirements, critical care interventions and length of stay. Severity scoring systems would also be useful if they were able to predict the risk of viral shedding/nosocomial infection and the consequent level of personal protective equipment required. Above all they need to be simple and based on widely available clinical and laboratory factors if they are to be used routinely. In this study patients were graded by all three severity systems, with the SSI system aligning best to the 39/41 in the moderate/severe group. The comparative mild severity group was identified by all three scoring systems designating the participant as mild/low risk.

Clinical and laboratory features at admission are important for diagnosis, surveillance and incorporating into severity scores, but limited data exists on the natural history/evolution of CCHF and other viral haemorrhagic fevers. There were significant differences in a range of laboratory variables between the moderate/severe and mild groups. The median platelet count at admission for the cohort was 104 x 10^9/L, with median liver enzyme levels and CK only mildly elevated to 2 x the upper limit of normal. This study is unique in being able to describe the longitudinal clinical and laboratory features of CCHF collected through a standardised case record form. The most frequent symptoms reported were headache, myalgia, lethargy and vomiting, all occurring in most patients for 2-3 days. In total 23% of the cohort had an episode of bleeding, that lasted for a median 2 days.

Duration of fever was surprisingly shorter in the moderate/severe group than in the mild group, with different temperature slopes demonstrated in linear analysis. Heart rate also reduced during the course of illness, and as might be expected the moderate/severe group had a lower systolic blood pressure and higher respiratory rate elevation/intercepts. Overall review of the vital sign data for the cohort demonstrates that in the majority of cases, a significant systemic inflammatory/sepsis response is not evident. It is also important to highlight that whilst approximately 25% of patients had bleeding, this was not in conjunction with multi-organ failure or disseminated intravascular coagulation (DIC). Patients with haemorrhage had normal vital signs, probably highlighting why CCHF is regularly transmitted nosocomially to non-infection specialists such as ENT surgeons whilst nasal packing for epistaxis. This robust evaluation of vital signs and severity is important as it provides the first clear evidence that a significant percentage of the CCHF managed in Turkey is 'mild' consistent with the lower-case fatality rates reported. It also provides the first indication and evidence that the bleeding pattern and coagulopathy observed is not simply due to DIC, subsequently further evaluated through ROTEM analysis.

There were clear patterns in some laboratory variables. Thrombocytopenia is a consistent feature in CCHF and has been shown to be associated with mortality [180]. A number of mechanisms that lead to the thrombocytopenia seen in CCHF have been suggested, and as well as their key roles in maintaining haemostasis, platelets are now increasingly recognised as major inflammatory cells with roles in both the innate and adaptive immune systems [193,194]. The rise in liver enzymes is consistent with dissemination of infection and then viral replication within hepatocytes: fatal cases of fulminant liver failure occurring in CCHF have been reported [157,188]. Renal dysfunction was not commonly seen in this cohort of CCHF with only 4/104 having acute kidney injury by RIFLE criteria. Although rare, the aetiology of renal dysfunction requires further evaluation to understand how much is a result of direct viral renal damage, renal hypovolemia due to sepsis or as a consequence of DIC. The main limitations in the clinical and laboratory description of the natural history of CCHF are that a smaller number of fatal and severe cases were recruited than planned. This limits the ability to improve understanding of the more complicated spectrum of CCHF disease, but unlike other reports does describe a large cohort of 'mild' disease for comparison.

8.2 Case management

The case management of CCHF is largely supportive with a focus on the provision of blood component therapy to prevent bleeding and critical care support when it is required for severe cases. Supportive care of patients during the study was guided by infectious diseases physicians in the different sites, with the vast majority receiving ward level care. Only 2 patients received

additional critical care support, both in the final stages of illness and lasting less than 24 hours. This further confirms the high percentage of 'mild' CCHF seen in Turkey.

Blood components were given to 37% of participants in the study and receipt of blood products was associated with disease severity. Although this would be expected this has not been reported previously when scoring systems were independently validated. Blood products were also utilised in patients that were graded as mild/low risk by all severity systems, demonstrating the limitations of severity scores at admission to be able to predict the need for transfusion. Platelet transfusion was generally administered according to absolute platelet count, with virtually all participants with counts <50 x 10^9/L and all patients with counts <20 x 10^9/L receiving platelet transfusion. The use of FFP was more variable and could be rationalised with more protocolised care.

The evaluation of ribavirin in CCHF has mostly been in retrospective studies: the one randomised controlled trial failed to show any reduction in mortality. A subsequent metal-analysis [173] did not demonstrate that ribavirin conferred any survival benefit or additional benefit such as shorter hospital stay, earlier improvement of laboratory values or decreased requirement for blood products. The utility of ribavirin in post-exposure prophylaxis (PEP) for healthcare workers that have a had a high risk CCHFV exposure such as a needle stick injury is much clearer [175]. Ribavirin was administered to 35% of participants, all of whom were managed at Gaziosmanpasa University Hospital. It was started a median of 4 days after onset of symptoms and administered for a median 5 days. However matched viral load analysis showed that 25% of participants had already cleared CCHFV from blood when it was started. Participants had also cleared CCHFV from blood for 72% of the days on which it was administered. Ribavirin has an established dose-dependent side effect profile and although its benefit needs to be established in large randomised studies, improved access to PCR based CCHFV monitoring could reduce its use and side effects.

8.3 Viral load (aim 1 &2)

This is the largest study of CCHFV viral load undertaken to date, with most previous studies evaluating CCHFV viral loads in small patient cohorts at baseline, with the exception being Hasanoglu et al [99] who reported baseline viral loads on 126 patients over 6 years. Similarly to that and earlier smaller studies, we demonstrated higher CCHF viral loads in fatal cases compared to survivors. We also demonstrated for the first time that there is a higher viral load in moderate/severe cases compared to mild cases using a composite severity scoring system.

In Turkey, a key variable that influences patient referral to CCHF specialist centres is platelet count. We have shown for the first time that participants that dropped their platelet counts to

less than 50 x 10^9/L had significantly higher CCHF viral loads at admission than those that did not. The laboratory parameters of APTT, PT, LDH, CK and creatinine also correlated with CCHF viral load, as did the duration of clinical features: fever; myalgia and vomiting. We have also demonstrated a correlation between CCHF viral load and bleeding during admission and also shown that viral load correlates with the use of blood products.

Longitudinal viral loads of any viral haemorrhagic fever are rarely measured with hypotheses about viral dynamics in blood often crudely inferred from admission sampling at different time points. A few small studies have undertaken this in CCHF, the largest of which was by Bodur et al [100] that investigated VL dynamics in plasma in a case-control study in a cohort of 50 patients. This is the first study to show that there is clear differences in CCHF viral dynamics in plasma by disease severity, including in mild disease and in patients who develop platelet counts to less than 50 x 10^9/L. The majority of patients cleared CCHFV from blood within 5-6 days of onset of symptoms: this has important infection prevention and control implications and is relevant when considering antiviral treatment and planning for future clinical trials, that may use CCHFV clearance as primary or secondary end-points.

The study was not designed to investigate the potential effect of ribavirin on CCHFV, and when it was initially designed ribavirin was not utilised by the study sites. However, after extension to another tertiary specialist centre, one third of the total cohort received ribavirin treatment. The use of ribavirin in CCHF has been a controversial issue, with no strong recommendations for use given by WHO, CDC or ECDC. Published meta-analyses are limited by retrospective observational data sets, with the most recent Cochrane evaluation reporting that that there was insufficient evidence to show whether it is effective in treating CCHF, and recommending randomised clinical trials [174]. Even allowing for uncertainty about the effectiveness of ribavirin the inclusion of patients who received ribavirin has the potential to alter the natural history of disease and viral dynamics in this cohort. It does however provide an opportunity to observationally evaluate ribavirin treatment, whilst recognising that although not statistically significant, those with moderate/severe disease were more likely to receive it due to the referral mechanisms in place in Tokat.

Viral elimination slopes were compared through linear regression for those that receiving ribavirin and those that did not (Figure 4.5). This is a binary classification of the two groups and fails to account for a significant percentage of patients who were initially managed without ribavirin, and then it received it on transfer to a tertiary facility. This has the potential to underestimate the effect of ribavirin on CCHFV slopes, and may explain the difference in figures 4.5 and 4.7. Figure 4.7 does show a difference between the typical patient model when the ribavirin data is evaluated by day of onset against the non-ribavirin typical patient. One of the

limitations of the ability to demonstrate a benefit of ribavirin therapy has been the low case fatality rate in Turkey, that means that a future RCT would require study arms of 600 for adequate power. However utilising different primary end-points such as viral clearance and elimination slope would be more feasible, assuming ribavirin has a direct antiviral effect. Daily viral load analysis prior to and during ribavirin therapy does however provide the first practical evaluation of its use in normal clinical practice in Turkey.

There is limited data on the viral dynamics of CCHFV in urine, but a previous report from a small cohort in Kosovo suggested evidence of prolonged viraemia in blood and urine [103]. As these were interesting results daily urine sample collection was undertaken during admission and again at days 14 and 30 follow-up. In total, 679 urines were processed from the 104 participants, including 54 samples at day 14 and 47 samples at day 30. All convalescent samples were PCR negative, and only 8/578 acute admission urine samples were PCR positive, all from different participants. It is clear from these results that urine is rarely CCHFV PCR positive in acute infection, and cannot be reliably utilised in convalescence to diagnose recent CCHF infection. The urine and plasma results are noticeably different from the results reported from Kosovo. However, the total number of urines evaluated from confirmed patients is not reported and the samples were not taken as part of a prospective study. The pattern of CCHF clinical disease reported from colleagues in Kosovo may also different than that seen in Turkey. A future prospective study from Kosovo utilising the same RT-PCR, with matched serum results is required to try to re-produce these preliminary results, and to allow further investigation of the different results observed.

In summary, this intensive evaluation of CCHF viral load has shown higher levels by markers of disease severity including platelet count and patient outcome. Viral load has been shown to correlate with clinical features such as bleeding and duration of fever and myalgia, and also with other laboratory variables such as APTT, LDH and CK, with a delayed correlation with platelet count observed. Longitudinal analysis of viral dynamics has again shown difference by CCHF disease severity, with earlier clearance observed in mild cases. For the first time a potential effect of ribavirin on plasma viral elimination slopes has been shown. Contrary to a previous report the presence of CCHFV in urine is rare.

8.4 ELISA antibody responses (aim 3)

The adaptive immune responses to CCHFV have not been well characterised previously. Improved data on the antibody responses to infection is critical for understanding the natural history of infection and has implications for both current diagnosis and the future development of diagnostics and vaccines. Development and testing of commercial serology assays for detecting IgM and IgG antibodies to CCHFV for use in supplementary diagnostic tests, for

epidemiology and surveillance during outbreaks, and for evaluation of vaccine immunogenicity and durability have also been identified as a priority research area by WHO [267].

This is the largest and most complete study of IgM and IgG antibody responses to acute infection with CCHFV to date , both in terms of sample size and the total number of samples analysed due to serial daily sampling. It is also the first time that antibody profiles in a cohort of patients with acute CCHF has been followed up at 14, 30 and 365 days. In total, over 750 samples from the cohort of PCR confirmed CCHF were analysed for CCHFV IgM/IgG by a commercial ELISA, VectoCrimean-CHF-IgM/IgM produced by Vectorbest. At admission 40% of patients had a positive IgM result, with 30% of patients being both IgM and IgG positive. Participants became IgM positive a median of 5 days into illness and all samples tested after 8 days of clinical illness were shown to positive for IgM. IgM remained positive in all convalescent samples tested, although titres were significantly lower at 30 days in comparison to 14 days. At admission just over 50% of participants had a positive CCHFV IgG result, with the median time for IgG seroconversion again being 5 days. After 8 days of clinical illness 96% of acute samples tested were also positive for IgG.

There was no significant difference in IgM/IgG titres at admission or convalescence when stratified by CCHF disease severity, or fatal or surviving cases. The distribution of CCHF viral load by different antibody groups was significantly different. The aetiology of disease in CCHF PCR negative suspect patients is unclear with limited diagnostic 'fever panels' being utilised in this cohort. They may include patients with CCHF who have cleared CCHFV from blood and some units send serum for IgM testing at the time of their discharge. In this study the CCHF PCR negative participants underwent IgM/IgG analysis in PHE and 3/39 participants were found to be IgM and IgG positive, with 6/39 participants CCHFV IgM negative/IgG positive.

There is limited indirect evidence or knowledge about antibody responses in other viral haemorrhagic fevers to compare with CCHF. In Lassa fever, little is known about the immune control of acute infection and recovery has not been shown to correlate with IgG/IgM production [213,268], with neutralizing antibodies only detected after clinical recovery [214]. There is consistent with animal model data such as a study of Lassa fever in cynomolgus macaques by Baize at al [215], that failed to detect neutralising antibodies in up to month after infection in survivors. They did however notice that LV-specific IgM and IgG appeared at earlier stage in survivors.

These data is in accordance with the previous data sets reported but has evaluated CCHF antibody responses in much greater depth. The ability to detect differences between fatal and surviving cases is probably due to a lack of statistical power as a result of the small number of fatal cases recruited. The data are broadly similar to those reported in EVD [216–218] with early IgG

responses, and persistence of IgM response also seen in dengue [219]. In summary the evaluation of the antibody responses in CCHF utilising the Vectorbest kit, is the most detailed and complete undertaken to date. It provides important insights into timing and progression of IgM/IgG responses to CCHFV with important diagnostic implications and combined with the PCR data, further validates this commercial test for wider use, that is a landmark goal of the WHO R&D blueprint.

8.5 ROTEM analysis

This study is the first to investigate the use of a global haemostasis test, ROTEM, alongside conventional testing in CCHF, demonstrating that using ROTEM is feasible and can provide a safe rapid point of care capability. Haemorrhage frequently occurs in severe disease and traditional laboratory tests (PT/APTT) do not show dramatic changes [100,160,180]. This suggests that the coagulopathy seen in patients with CCHF is not due to DIC but may lie in areas not measured by conventional coagulation testing, such as a defect in platelets or fibrinolysis.

In this large cohort of patients in Turkey we demonstrate new insights into the aetiology and natural history of the coagulopathy in CCHF. Patients presented after a median of two days illness with a median platelet count of 73 x 10^9/L, and mean PT and APTT of 14 s and 38.3 s respectively, i.e. both coagulation tests within the normal range and yet patients had a bleeding tendency by ROTEM analysis. At admission, the majority of patients had normal EXTEM S clotting times (CT), showing that initiation of clotting, thrombin formation and the start of clot polymerisation was not significantly abnormal in most cases at this point. In contrast, EXTEM S CFTs were abnormal indicating problems with fibrin polymerisation and stabilisation of the clot with Factor XIII and platelets. In the face of normal or slightly deranged APTT/PT, this points to a platelet and/or Factor XIII defect. Activation of fibrinolysis can be discounted as there was no significant reduction in MCF/clot lysis.

Our results show that the vast majority of patients at admission had normal FIBTEM results, indicating normal fibrinogen levels. A key finding in DIC is the presence of hypofibrinogenaemia and there is also consumption of coagulation factors with the formation of small thrombi throughout the vasculature leading to end organ failure. The absence of significantly low fibrinogen levels or of activation of fibrinolysis are not in keeping with DIC. We did detect a reversible coagulopathy in those who survived CCHF and when acute samples were stratified by day of illness, values were most abnormal on days 4-6, indicating that this is the major coagulopathic period in CCHF with respect to a patient's ability to generate stable clots.

In summary, we have shown a coagulopathy developed during CCHF infection, that was not associated with a major coagulation defect as evidenced by only minor changes in PT, APTT, and

ROTEM clotting time and FIBTEM. Importantly, ROTEM has shown no evidence of hyperfibrinolysis. Such a defect could be due to either Factor XIII deficiency or a platelet dysfunction, to account for the bleeding and the defect in clot firmness. We plan to investigate Factor XIII further but consider it unlikely to be the cause of the bleeding defect. The clinical pattern of bleeding is also in keeping with a platelet defect as bleeding was predominantly from mucous membranes.

Platelet dysfunction has been shown in other viral haemorrhagic fevers, particularly by Cummins et al, who showed a clear platelet function defect on platelet aggregometry in both Lassa and Argentinian HFs [184,228]. Our findings exclude DIC as the cause of the bleeding defect in all patients at presentation with CCHF. However we do recognise that DIC can occur in fatal cases of CCHF associated with multi-organ failure. The main limitation of the ROTEM analysis was that less patients with severe/fatal disease and with haemorrhage were recruited than expected, but we were able to demonstrate that the coagulopathy occurred in those graded with mild disease. ROTEM analysis was also not routinely undertaken at specific time points after transfusion of blood products to allow evaluation of effect on haemostasis, and we plan to evaluate this in future studies. We would have liked to evaluate the prognostic and predictive effect of ROTEM parameters for the development of bleeding and use of blood products, but due to the study design and individual clinician-based choice for utilisation of blood products this was not possible.

In summary, ROTEM and conventional coagulation analysis has demonstrated that CCHF coagulopathy is predominantly related to clot development/stabilisation that is most marked during day 4-6 of the illness and in severe disease. In the context of normal/only slightly deranged coagulation screens and FIBTEM results, and combined with the absence of hyperfibrinolysis, this data provides the first evidence that the initial coagulopathy in CCHF probably relates to platelet dysfunction.

8.6 Cytokines/chemokines (aim 3&4)

Cytokines are key proteins involved in the host immune response, that have both pro-inflammatory and immunomodulatory roles. Regulation of the cytokine network maintains the equilibrium and sepsis and related organ dysfunction are frequently attributed to excessive release of pro-inflammatory cytokines. The majority of studies published to date investigating cytokine expression in CCHF have been based on admission samples and do not reveal the longitudinal trend of cytokine expression in acute illness.

This study evaluated the dynamic cytokine response through intensive daily sampling and utilised a comprehensive 19 plex human cytokine panel in 102 participants with confirmed CCHF. Samples were also rapidly processed and frozen to prevent degradation, with no freeze-thaw cycles prior to first analysis and were measured in duplicate. We also recruited a significant cohort 'mild' severity CCHF and followed participants to 30 days after onset of symptoms.

Evaluation of the individual cytokines at admission clearly demonstrates that a number are statistically significant, independent of the method utilised for stratifying groups for comparison. These include IL-10, IL-15, IL-6, IL-8, IP-10, MIP-1α and TNF-α, . However, IL-1β, IL-2, IL-5 and IL-17A had levels that remained low and at the limit of detection, with no difference in convalescence samples. No difference was also noted in these cytokines at admission when stratified by disease severity or outcome, suggesting that they are not playing a key role in CCHF pathogenesis. IL-1B in particular was expected to demonstrate more significant results as it is considered an important pro-inflammatory cytokine. We had hypothesised that elevated levels would be associated with severe disease as Papa et al [123] had previously found it to be raised in fatal cases. The elevated levels of the remaining 15 cytokines/chemokines analysed, compared to convalescence levels suggest a role in the complex immune response to CCHFV infection. However, differences in cytokine profiles when they are stratified by markers of disease severity provides more interesting data.

Consistent with previous reports, we have demonstrated that at admission the cytokines IL-10, IL-6, IP-10 MCP-1, TNF-α are significantly higher in moderate/severe vs mild cases. However, for the first time GM-CSF, IL-12, IL-4 and IL-8 have been shown to be higher in moderate/severe cases and furthermore when severity is gauged by platelet count, MIP-1α, MIP-1β IL-15, INFA2 and G-CSF are also elevated in patients who have platelet counts <50 x 10^9/L. Stratification by outcome (fatal/survived) revealed differences in IL-10, IL-6 and TNF-α levels in line with published reports. We also found further significant associations between higher levels of IL-15, IL-8 and MCP-1 and mortality despite only a small number of fatal cases.

The opportunity to evaluate cytokine profiles with matched CCHF viral load data is rarely available. We demonstrated correlations between admission cytokines and viral load for a range of cytokines/chemokines including TNF-α and IL-6, but not for IP-10, IL-10 or IFN-γ. However, when the dataset was expanded to evaluate correlations in all CCHFV positive longitudinal samples IL-10 and IP-10 positive correlations were demonstrated. The other cytokines that correlated with viral load at admission were IFN-A2, IL-15, IL-8 and MIP-1β. Longitudinal analysis of cytokine expression highlights linear trends during illness course with predominant increased pro-inflammatory immune responses associated with severe disease and death, particularly in G-CSF, IL-6, IL-8, IL-15, MCP-1, MIP-1α, MIP-1β and TNF-α. IL-10 is a key anti-inflammatory cytokine that remains elevated in severe and fatal disease.

The cytokine profiles that we have identified in patients with CCHF represent a valuable tool for the characterisation of immunological response patterns. They highlight a complex relationship of feedback loops, with a range of cytokines, including some not analysed and other factors involved. The immune response to CCHF is not a simple model and our results suggest that both pro-inflammatory and anti-inflammatory responses correlate with the pathogenesis of sepsis in a combined relationship.

These data has for the first time also shown the expression of different cytokines during the course of illness and clearance of CCHF. It has identified additional biomarkers for disease severity and due the comprehensive, has highlighted the key cytokines involved in the host immune response. Immunomodulation of this response has to date not been shown to be beneficial in sepsis clinical trials, but this is likely to reflect the heterogenous nature of the sepsis syndrome. Whilst the priority for future clinical research in CCHF should focus on evaluating and antiviral/immune based therapeutics, there may also be potential in targeting specific steps of the interactive and dynamic process of the cytokine expression to limit organ dysfunction.

8.7 Limitations of the study

The main limitations of the study are the limited numbers of severe and fatal CCHF cases that were recruited, that was out with the control of the chief investigator. Due to informed consent procedures there was also an unavoidable delay in recruitment and sampling of participants. Due to quicker than predicted clearance of CCHFV from bloods, this had a direct effect on the number of participants with positive viral load data at entry of the study for comparison.

Recruitment of 'mild' severity CCHF disease is important to improve understanding of host immune response and viral dynamics, and the study recruited from secondary and tertiary health care facilities. Seroprevalence studies in Turkey have though highlighted high rates of CCHFV IgG positivity in populations living in hyperendemic areas that is thought to represent an undiagnosed/asymptomatic infection. To fully investigate this group/spectrum of illness further a combination of prospective fever and longitudinal epidemiological studies are required.

There is a potential effect of ribavirin use in one third of the cohort, that whilst providing important observational data, may have had a confounding effect on viral loads, particularly when analysed by disease severity. There were also different times to presentation and a small number of participants who were transferred to study sites from other facilities. The cohort recruited in Tokat and Samsun are broadly representative of the adult CCHF population in Turkey, but host and viral genetic factors in other endemic countries such as Iran, Kosovo and South Africa must be considered when considering the results in a broader context. Data was collected by trained study teams at each site, but it is possible that errors were made in CRF completion and sample labelling although great care was made to avoid this. Data was entered and independently checked, then verified.

Whilst recognising these limitations it must also be recognised that undertaking an intensive clinical and laboratory study of this nature in a large cohort of patients with viral haemorrhagic fever, presents significant IP&C and safety challenges throughout that must be mitigated.

8.8 Conclusions and summary key points

This comprehensive study of a large cohort of adults with confirmed CCHF in Turkey has described the clinical features and management, immune responses and viral dynamics providing new insights into disease pathogenesis. For the first time a large number of 'mild' severity CCHF cases have been included that provide an important group for comparison, particularly with respect to host immune response to CCHF infection. ROTEM analysis has been undertaken at scale for the first time in any viral haemorrhagic fever and combined with standard laboratory tests and clinical observation, highlighted a mechanism for the coagulopathy and pattern of bleeding seen.

- A high percentage of patients admitted with confirmed CCHF in Turkey have mild severity disease (60%), with the majority recording vital signs within normal limits at admission and during hospitalisation.
- Consistent with other VHFs, high fever is not characteristic of CCHF with important implications for case definitions utilised in surveillance and screening.
- Thrombocytopenia and a hepatitis are consistent features of CCHF and associated with disease severity and higher CCHF viral loads. Renal dysfunction is not commonly seen in CCHF with only 4% having acute kidney injury by RIFLE criteria.
- Bleeding occurred in 23% and blood component therapy was received by 37% of participants, associated with increased disease severity.
- Ribavirin was administered to 35% of participants, a median of 4 days after onset of symptoms. 25% of participants were CCHF PCR negative when ribavirin was commenced and for 72% of the days it was administered.
- Higher CCHF viral load is associated with increased disease severity (mild vs moderate/severe), lower platelet counts and fatal outcome. The laboratory parameters of APTT, PT, LDH, CK and creatinine correlated with CCHF viral load, as did the duration of clinical features: fever; myalgia and vomiting.
- Most patients clear CCHFV from blood within 5-6 days of onset of symptoms, occurring more rapidly in patients with mild disease and higher platelet counts (65% CCHFV clearance at day 5 of illness vs 40% in moderate/severe group). Urine is rarely CCHFV PCR positive in acute infection (8/578 samples) and cannot be reliably utilised in convalescence to diagnose recent CCHF infection.
- Duration of viraemia positively correlated with a longer length of hospital stay and number of platelet transfusions required.

- At admission, 42% of patients are IgM positive, with all patients IgM positive by day 8 of clinical illness continuing to 30 days. At admission, 50% of patients are IgG positive, with >95% positive by day 8 of illness.
- ROTEM and conventional coagulation analysis has demonstrated that the coagulopathy in CCHF is reversible and predominantly related to clot development/stabilisation that is most marked during day 4-6 of the illness and in severe disease. In the context of normal/only slightly deranged coagulation screens and FIBTEM results, and combined with the absence of hyperfibrinolysis, this data provides the first evidence that the initial coagulopathy in CCHF probably relates to platelet dysfunction and not DIC.
- In accordance with previous data, at admission the cytokines IL-10, IL-6, IP-10, MCP-1, TNF-α were significantly higher in moderate/severe vs mild cases. However, for the first time GM-CSF, IL-12, IL-4 and IL-8 were shown to elevated in moderate/severe disease.
- Stratification by outcome (fatal/survived) also revealed differences in IL-10, IL-6 and TNF-α levels in line with published reports. We also found further significant associations with mortality and higher levels of IL-15, IL-8 and MCP-1 despite a small number of fatal cases.
- Significant linear regression slopes during the course of illness were demonstrated by IFN-α2, IL-5, IL-10, IL-15, MCP-1, MIP-1α, MIP-1β and TNF-α. At admission we demonstrate previously reported correlations with TNF-α and IL-6 with CCHF viral load, and also with IFN-A2, IL-15, IL-8 and MIP-1β.

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Appendix 1

Ministry of Defence Research Ethics Committee

From the Chairman
Professor Allister Vale MD
National Poisons Information Service (Birmingham Unit),
City Hospital, Birmingham B18 7QH

Telephone: 0121 507 4123
e-mail:allistervale@npis.org

Major Thomas Fletcher
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Our Reference:
571/MODREC/14

Date: 4 April 2015

Dear Major Fletcher,

Pathogenesis of Crimean-Congo Haemorrhagic Fever (CCHF) – Interaction of Immune Response, Viral Load and Clinical Course

Thank you for submitting your revised Application 571 with tracked changes, and with a covering letter with responses to my own letter. The revised protocol has been approved by the Officers of MODREC ex-Committee, subject to *a number of minor further changes shown in red, including a change to the Participant's statement.* If you agree with these further changes please confirm this to the Secretariat, copied to me.

I wish you and your colleagues a successful study. In due course please send the Secretariat a final report containing a summary of the results so that these can be filed in accordance with the arrangements under which MODREC operates**.** Please would you also send a brief interim report in one year's time if the study is still ongoing.This approval is conditional upon adherence to the protocol – please let me know if any amendment becomes necessary.

Yours sincerely

Allister Vale

Allister Vale MD FRCP FRCPE FRCPG FFOM FAACT FBTS FBPhS FEACCT Hon FRCPSG

cc, Professor David Jones, Professor David Baldwin, Ethics Secretariat

Appendix 2

T.C.

ONDOKUZ MAYIS ÜNİVERSİTESİ

KLİNİK ARAŞTIRMALAR ETİK KURULU

Sayı: B.30.2.ODM.0.20.08/ 1154

25.07.2014

Sayın : Prof Dr Hakan LEBLEBİCİOĞLU

Etik Kurulumuza sunmuş olduğunuz **Kırım Kongo Kanamalı Ateşin Patogenezi-Bağışıklık yanıtı, viral yük ve klinik seyrin etkileşimi** başlıklı OMÜ KAEK 2014/ 739 Karar nolu Mikrobiyoloji çalışması nitelikli araştırma projeniz: Amaç, gerekçe, yaklaşım ve yöntemle ilgili açıklamaları, Klinik Araştırmalar Etik Kurulu yönergesine göre 24 .07.2014 tarihli Etik Kurulumuzda incelenmiş etik açıdan uygun bulunmuştur. Ancak araştırma bütçesinin maddi desteği henüz sağlanamadığından projeye bütçe desteği sağlanıp, tarafımıza bildirilmesinden sonra *başlanmasına* oy birliği ile karar verilmiştir.

Bilgilerinize arz/rica ederim.

Prof.Dr.Abdulkerim BEDİR
Klinik Araştırmalar Etik Kurulu
Başkanı

Ondokuz mayıs Üniversitesi Tıp Fak. Klinik Araştırmalar Etik Kurulu Tel:(0362)3121919/2782 -4576007 Omutaek@gmail.com
Hastane içi 1.Kat (Özel servis karsısı) Atakum/SAMSUN

Appendix 3

World Health Organization ISARIC

CASE RECORD FORM INSTRUCTIONS

COMPLETE CRIMEAN CONGO HAEMORRHAGIC FEVER DATASET

This form includes all available modules of the COMPLETE CCHF DATASET.

Modules included in each set of forms are:

ADMISSION	DEMOGRAPHICS
	SIGNS AND SYMPTOMS
	PATIENT INFORMATION - CASE INVESTIGATION
	EPIDEMIOLOGICAL RISK FACTORS AND EXPOSURES - CASE INVESTIGATION
	CO-MORBIDITIES
	PRE-ADMISSION MEDICATIONS
	EXPOSURE
DAILY	DAILY OBSERVATIONS & TREATMENTS
	DAILY LABORATORY RESULTS
	MEDICATIONS & BLOOD PRODUCTS
	VIRUS TESTING
	OTHER INFECTIONS
	CRITICAL CARE
OUTCOME	SEVERE SYMPTOMS & COMPLICATIONS
	OUTCOME
	PATIENT OUTCOME INFORMATION - CASE INVESTIGATION
	FOLLOW-UP

GENERAL GUIDANCE

- Patient numbers consist of a 3-digit site code and a 4 digit patient number. You will be assigned a site code or can obtain a site code by registering with the data manager at isaric@oucru.org. Patient numbers should be assigned sequentially for each site beginning with 0001. In the case of a single site recruiting patients on different wards, or where it is otherwise difficult to assign sequential numbers, it is acceptable to assign numbers in blocks, e.g. by ward where Out-patient ward will assign numbers from 0001 onwards. In-patient ward will assign numbers from 5001 onwards. Alpha characters can also be used. E.g. Out-patient ward will assign A001 onwards. In-patient ward will assign B001 onwards. **Please enter the unique patient identification code at the top of each and every paper sheet.**
- **Complete every line of every section**, except for where the instructions say to skip a section based on certain responses.
- Selections with circles (O) are single selection answers (choose one answer only). Selections with square boxes (☐) are multiple selection answers (choose as many answers as are applicable).
- It is important to know when the answer to a particular question is not known. Please mark the 'Unknown' box if this is the case. For laboratory values, please enter "NA" in the data space when results are Not Available.
- We recommend writing clearly in black or blue ink, using BLOCK-CAPITAL LETTERS.
- Place an (X) when you choose the corresponding answer. To make corrections, strike through (-------) the data you wish to delete and write the correct data above it. Please initial and date all corrections.
- Please keep all of the sheets for a single patient together e.g. with a staple or in a folder that is unique to the patient.
- **Please observe your local infection control policy on record keeping and movement of records in/out of clinical areas.**

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

DEMOGRAPHICS

Tarih: *(DD/MM/YYYY):* [____][____]/[____][____]/[__2_][__0_][____][____]

Soy isim:_____ **Isim:**_____

Klinik odi:_____

il: _____

ilae: _____

Telefon No:

Yakininin Adi ve telefonu

Hostaneye Basuuru Tarihi *(DD/MM/YYYY)*: [____][____]/[____][____]/[__2__][__0__][____][____]

Cinsiyet: O Erkek O Kadin **Dopum Tarihi** *(DD/MM/YYYY):* [____][____]/[____][____]/[____][____][____][____]
OR
Tohmini yas [___][___][___] O years O months *(<2 years)*

KKKA siniflamasi: O PCR + O Supheli O Bilinmiyor

Known Tick exposure: O Yes O No Date: _____

: Is the patient: O Pregnant O Gave birth within previous 6 weeks O Neither Unknown

If PREGNANT: Gestation age of fetus *(nearest week)*: [___][___]

If GAVE BIRTH WITHIN 6 WEEKS: Pregnancy Outcome: O Live birth O Still birth O Termination
O Spontaneous abortion/miscarriage O Unknown

HOSPITALIZATION INFORMATION

Was the patient hospitalized or did he/she visit a health clinic previously <u>for this illness</u>? O YES O NO O Unknown

If yes, please complete a line of information for each previous hospitalization:

Dates of Hospitalization *(DD/MM/YYYY)*	**Health Facility Name**	**District**	**Was the patient isolated?**
__ __/__ __/20__ __ to __ __/__ __/20__ __			O YES O NO O Unknown
__ __/__ __/20__ __ to __ __/__ __/20__ __			O YES O NO O Unknown

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

SIGNS AND SYMPTOMS *(first available data at presentation/admission – within 24 hours):*
Date: *(DD/MM/YYYY):* [____][____]/[____][____]/[__2_][__0_][____][____]

Maximum Temperature: [___][___][___].[___]O°C *or* O°F

Heart Rate: [___][___][___]beats per minute **Respiratory Rate:** [___][___]breaths per minute

Systolic Blood Pressure: [___][___][___]mmHg **Diastolic Blood Pressure:** [___][___][___]mmHg

Saturations: [___][___] % **FiO_2** *(0.21-1.0):* [___].[___][___] or [___][___]L/min

Date of onset of first/earliest symptom *(DD/MM/YYYY):* [____][____]/[____][____]/[__2__][__0__][____][____]

Signs and symptoms observed during this illness episode (between symptom onset and facility admission):

Fever	O YES	O NO	O Unknown	Back pain	O YES	O NO	O Unknown
Lethargy/asthenia	O YES	O NO	O Unknown	Chest pain	O YES	O NO	O Unknown
Headache	O YES	O NO	O Unknown	Sore throat	O YES	O NO	O Unknown
Joint or muscle pain/aches	O YES	O NO	O Unknown	Conjunctival injection	O YES	O NO	O Unknown
Loss of appetite	O YES	O NO	O Unknown	Skin rash	O YES	O NO	O Unknown
Difficulty swallowing	O YES	O NO	O Unknown	Decreased urine output	O YES	O NO	O Unknown
Nausea	O YES	O NO	O Unknown	Cough	O YES	O NO	O Unknown
Vomiting	O YES	O NO	O Unknown	Confused/disoriented	O YES	O NO	O Unknown
Diarrhoea	O YES	O NO	O Unknown	Seizures	O YES	O NO	O Unknown
Abdominal pain	O YES	O NO	O Unknown	Peripheral oedema	O YES	O NO	O Unknown
Hiccups	O YES	O NO	O Unknown	Abdominal tenderness	O YES	O NO	O Unknown
Breathing difficulty	O YES	O NO	O Unknown	Jaundice	O YES	O NO	O Unknown
Bleeding. If YES, specify:	O YES	O NO	O Unknown	Hepatomegaly	O YES	O NO	O Unknown
Epistaxis/nose	O YES	O NO	O Unknown	Splenomegaly	O YES	O NO	O Unknown
Gingival/oral	O YES	O NO	O Unknown	Petechiae	O YES	O NO	O Unknown
Coughing up blood	O YES	O NO	O Unknown	Bruising	O YES	O NO	O Unknown
Fresh red blood in vomit	O YES	O NO	O Unknown	Other symptom	O YES	O NO	O Unknown
Brown blood in vomit (coffee grounds)	O YES	O NO	O Unknown	If YES, Specify Other:	_____		
Blood in urine	O YES	O NO	O Unknown				
Fresh red blood in stool	O YES	O NO	O Unknown				
Melaena blood in stool (tar black)	O YES	O NO	O Unknown				
Vaginal	O YES	O NO	O Unknown				
Line/venepuncture/injection site	O YES	O NO	O Unknown				
Other haemorrhagic symptoms	O YES	O NO	O Unknown				
If YES, Specify Other:							

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

CO-MORBIDITIES *(existing PRIOR TO ADMISSION & that are active problems)*			
Chronic cardiac disease	OYES ONO OUnknown	Metastatic solid tumour	OYES ONO OUnknown
Chronic pulmonary disease *(including TB, not including asthma)*	OYES ONO OUnknown	Any malignancy including leukaemia & lymphoma	OYES ONO OUnknown
Physician diagnosed asthma	OYES ONO OUnknown	AIDS / HIV	OYES ONO OUnknown
Renal disease	OYES ONO OUnknown	Obese as defined by clinical staff	OYES ONO OUnknown
Moderate or severe liver disease	OYES ONO OUnknown	Diabetes	OYES ONO OUnknown
Mild liver disease	OYES ONO OUnknown	Rheumatologic disease	OYES ONO OUnknown
Chronic neurological disease	OYES ONO OUnknown	Dementia	OYES ONO OUnknown
Hemiplegia or paraplegia	OYES ONO OUnknown		

PRE-ADMISSION MEDICATIONS - List all medications (antibiotics, antifungals, antivirals, anti-malarials, analgesics, antipyretics, etc) given for this illness episode PRIOR to presentation: *(add more pages if required)*

Name of medication *(generic name preferred)*	**Dose and frequency**	**Start date** *(DD/MM/YYYY)*	**End date** *(DD/MM/YYYY)*	**Route of administration**
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown
	Ounknown	__ __/__ __/20 __ __	On-going __ __/__ __/20 __ __	OIV Ooral Oinhaled Oother Ounknown

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

Complete as much as you can measure beginning one day after hospital/facility admission – information collected should reflect the previous 24 hour period. Enter minimal vital signs on Day 1, 3, 7, 14 and 28.

DAILY OBSERVATIONS AND TREATMENTS *Complete all with the (most abnormal) value in the previous 24 hours.*							
DATE: DD/MM YEAR 20__ __	__/__	__/__	__/__	__/__	__/__	__/__	__/__
Maximum Temperature O°C *or* O°F							
Respiratory Rate breaths/minute							
Heart Rate beats/minute							
Systolic Blood Pressure mmHg							
Diastolic Blood Pressure mmHg							
LOWEST Consciousness **A**lert, **V**erbal stimuli, **P**ainful stimuli, **U**nresponsive	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*
Lethargy/asthenia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Urine output Normal, Reduced	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**
Hypovolemia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Diarrhoea?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Vomiting/nausea?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Bleeding?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
IF YES Bleeding: *check/state all that apply* — **Epistaxis/nose?**							
Gingival/oral?							
In sputum?							
In vomit?							
In urine?							
In stool?							
Vaginal?							
Line/injection site?							
Other, Specify:							
Headache?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Cough?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Petechial Rash?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Arthralgia or myalgia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Other signs/symptoms?							
Since the last assessment, patient is:	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**	O**Improved** O**Stable** O**Worse**
In the last 24 hours, has the patient had:							
Intravenous fluids?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
IF YES, **IV fluid volume** Litres/24 hours							

UK = Unknown

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

DAILY OBSERVATIONS AND TREATMENTS *Complete all with the (most abnormal) value in the previous 24 hours.*							
DATE: DD/MM YEAR 20__ __	__/__	__/__	__/__	__/__	__/__	__/__	__/__
Maximum Temperature O°C *or* O°F							
Respiratory Rate breaths/minute							
Heart Rate beats/minute							
Systolic Blood Pressure mmHg							
Diastolic Blood Pressure mmHg							
LOWEST Consciousness **A**lert, **V**erbal stimuli, **P**ainful stimuli, **U**nresponsive	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*	*A V P U*
Lethargy/asthenia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Urine output Normal, Reduced	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**	O**Normal** O**Reduced**
Hypovolemia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Diarrhoea?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Vomiting/nausea?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Bleeding?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
If YES Bleeding: *check/state all that apply* — **Epistaxis/nose?**							
Gingival/oral?							
In sputum?							
In vomit?							
In urine?							
In stool?							
Vaginal?							
Line/injection site?							
Other, Specify:							
Headache?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Cough?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Petechial rash?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Arthralgia or myalgia?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
Other signs/symptoms?							
Since the last assessment, patient is:	O Improved O Stable O Worse	O Improved O Stable O Worse	O Improved O Stable O Worse	O Improved O Stable O Worse	O Improved O Stable O Worse	O Improved O Stable O Worse	O Improved O Stable O Worse
In the last 24 hours, has the patient had:							
Intravenous fluids?	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK	Yes No UK
IF YES, **IV fluid volume** Litres/24 hours							

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

DAILY LABORATORY RESULTS
Complete as much as you can measure. Critical values are listed at the top. Mark the correct unit where indicated. Use the most abnormal value per day. If Not Available enter "NA".

DATE: DD/MM YEAR 20__ __		__/__	__/__	__/__	__/__	__/__	__/__	__/__
↓ MOST CRITICAL →	**Sodium** mEq/L							
	Potassium mEq/L							
	Blood Urea Nitrogen Ommol/L *or* Omg/d							
	Creatinine Oμmol/L *or* Omg/dL							
	Chloride mEq/L							
	Bicarbonate mEq/L							
	Glucose Ommol/L *or* Omg/dL							
	Lactate Ommol/L *or* Omg/dL							
	Haemoglobin Og/L *or* Og/dL							
	Haematocrit %							
WBC count Ox10^9/L *or* Ox10^3/μL								
Lymphocyte count Ox10^9/L *or* Ox10^3/μL								
Platelets Ox10^9/L *or* Ox10^3/μL								
D-dimer Ong/mL *or* Omcg/mL								
APTT								
PT Seconds								
INR								
Fibrinogen								
Amylase U/L								
Bilirubin Oμmol/L *or* Omg/dL								
AST/SGOT U/L								
ALT/SGPT U/L								
Creatine kinase U/L								
Albumin g/dL								
CRP								

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

DAILY LABORATORY RESULTS
Complete as much as you can measure. Critical values are listed at the top. Mark the correct unit where indicated. Use the most abnormal value per day. If Not Available enter "NA".

DATE: DD/MM YEAR 20__ __		__/__	__/__	__/__	__/__	__/__	__/__	__/__
← MOST CRITICAL →	**Sodium** mEq/L							
	Potassium mEq/L							
	Blood Urea Nitrogen Ommol/L *or* Omg/d							
	Creatinine Oμmol/L *or* Omg/dL							
	Chloride mEq/L							
	Bicarbonate mEq/L							
	Glucose Ommol/L *or* Omg/dL							
	Lactate Ommol/L *or* Omg/dL							
	Haemoglobin Og/L *or* Og/dL							
	Haematocrit %							
WBC count Ox10^9/L *or* Ox10^3/μL								
Lymphocyte count Ox10^9/L *or* Ox10^3/μL								
Platelets Ox10^9/L *or* Ox10^3/μL								
D-dimer Ong/mL *or* Omcg/mL								
APTT								
PT seconds								
INR								
Amylase U/L								
Bilirubin Oμmol/L *or* Omg/dL								
AST/SGOT U/L								
ALT/SGPT U/L								
Creatine kinase U/L								
Albumin g/dL								

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

CRP							

MEDICATIONS: *List all medications administered from baseline to outcome.*				
Medication *(generic name preferred)*	**Volume or Dose and frequency** *(specify or unknown)*	**Start date** *(DD/MM/YYYY)*	**End date** *(DD/MM/YYYY)*	**Route of administration**
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown
	O unknown	__ __/__ __/20 __ __	O On-going __ __/__ __/20 __ __	O IV O oral O inhaled O other O unknown

BLOOD PRODUCT AND INTRAVENOUS FLUIDS: *List all administered from baseline to outcome*	**Volume or Units** *(specify or unknown)*	**Start date** *(DD/MM/YYYY)*
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __
	O unknown	__ __/__ __/20 __ __

CASE RECORD FORM – CCHF

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

CCHF TESTING:					
Sample Collection Date *(DD/MM/YYYY)*	**Local lab sample identifier (if available)**	**Sample Type**	**Method** *(one per line)*	**Test Kit Name** *(one per line)*	**Result**
__ __/__ __/20__ __		O **Blood** O **Other,** *Specify:* _____	O PCR O IgM O IgG O Other_____	Specify: _____	O Positive O Negative O Unknown
__ __/__ __/20__ __		O **Blood** O **Other,** *Specify:* _____	O PCR O IgM O IgG O Other_____	Specify: _____	O Positive O Negative O Unknown
__ __/__ __/20__ __		O **Blood** O **Other,** *Specify:* _____	O PCR O IgM O IgG O Other_____	Specify: _____	O Positive O Negative O Unknown

OTHER INFECTIONS: **Did the patient test positive for any other infection?** O YES O NO O Unknown If YES, Specify:			
Sample/Detection Date *(DD/MM/YYYY)*	**Local lab sample identifier**	**Sample Type**	**Pathogen (one per line)**
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____
____/____/20____		O **Blood** O **Other,** *Specify:* _____	*Specify:* _____

World Health Organization ISARIC

HASTA KAYIT NUMARISI:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

CRITICAL CARE *(Record the most abnormal value in the previous 24 hours)*:
Date: *(DD/MM/YYYY)*: [____][____]/[____][____]/[__2_][__0_][____][____]

Mechanical ventilation? O YES O NO **Glasgow Coma Score** *(out of 15)*: [___][___]

FiO_2 *(0.21-1.0)*: [___].[___][___] or [___][___]L/min

PaO_2 [___][___][___]O kPa *or* O mmHg

Oxygen saturation:[___][___][___]% Oxygen saturation on: O Room air O Supplemental Oxygen

Line in situ? O YES O NO If YES: O Peripheral O Central venous O Interosseous

Any vasopressor/inotropic support? O YES O NO **If YES, Select support:**
0 Dopamine <5µg/kg/min OR Dobutamine OR Milrinone OR Levosimendan: O YES O NO
1 Dopamine 5-15µg/kg/min OR Epinephrine/Norepinephrine < 0.1µg/kg/min OR vasopressin OR phenylephrine: O YES O NO
2 Dopamine >15µg/k/min OR Epinephrine/Norepinephrine > 0.1µg/kg/min: O YES O NO

CRITICAL CARE *(Record the most abnormal value in the previous 24 hours)*:
Date: *(DD/MM/YYYY)*: [____][____]/[____][____]/[__2_][__0_][____][____]

Mechanical ventilation? O YES O NO **Glasgow Coma Score** *(out of 15)*: [___][___]

FiO_2 *(0.21-1.0)*: [___].[___][___] or [___][___]L/min

PaO_2 [___][___][___]O kPa *or* O mmHg

Oxygen saturation:[___][___][___]% Oxygen saturation on: O Room air O Supplemental Oxygen

Line in situ? O YES O NO If YES: O Peripheral O Central venous O Interosseous

Any vasopressor/inotropic support? O YES O NO **If YES, Select support:**
Dopamine <5µg/kg/min OR Dobutamine OR Milrinone OR Levosimendan: O YES O NO
Dopamine 5-15µg/kg/min OR Epinephrine/Norepinephrine < 0.1µg/kg/min OR vasopressin OR phenylephrine: O YES O NO
Dopamine >15µg/k/min OR Epinephrine/Norepinephrine > 0.1µg/kg/min: O YES O NO

CRITICAL CARE *(Record the most abnormal value in the previous 24 hours)*:
Date: *(DD/MM/YYYY)*: [____][____]/[____][____]/[__2_][__0_][____][____]

Mechanical ventilation? O YES O NO **Glasgow Coma Score** *(out of 15)*: [___][___]

FiO_2 *(0.21-1.0)*: [___].[___][___] or [___][___]L/min

PaO_2 [___][___][___]O kPa *or* O mmHg

Oxygen saturation:[___][___][___]% Oxygen saturation on: O Room air O Supplemental Oxygen

Line in situ? O YES O NO If YES: O Peripheral O Central venous O Interosseous

Any vasopressor/inotropic support? O YES O NO **If YES, Select support:**
Dopamine <5µg/kg/min OR Dobutamine OR Milrinone OR Levosimendan: O YES O NO
Dopamine 5-15µg/kg/min OR Epinephrine/Norepinephrine < 0.1µg/kg/min OR vasopressin OR phenylephrine: O YES O NO
Dopamine >15µg/k/min OR Epinephrine/Norepinephrine > 0.1µg/kg/min: O YES O NO

CASE RECORD FORM – CCHF

World Health Organization ISARIC

PATIENT IDENTIFICATION NUMBER:
(3 digit site code – 4 digit sequential patient code) [__][__][__] - [__][__][__][__]

SEVERE SYMPTOMS & COMPLICATIONS:

Date: *(DD/MM/YYYY):*
[____][____]/[____][____]/[__2_][__0_][____][____]

At any time during hospitalisation did the patient experience:

Shock	O YES O NO O Unknown	IV line infection	O YES O NO O Unknown
Delirium/confusion	O YES O NO O Unknown	Acute renal injury/failure	O YES O NO O Unknown
Coma	O YES O NO O Unknown	Hepatic dysfunction	O YES O NO O Unknown
Seizure(s)	O YES O NO O Unknown	Hypoglycaemia	O YES O NO O Unknown
Pulmonary oedma	O YES O NO O Unknown	Hyperkalaemia	O YES O NO O Unknown
Bleeding	O YES O NO O Unknown	Hypokalaemia	O YES O NO O Unknown

If YES Bleeding, check all locations that apply:

☐ Epistaxis/nose ☐ Gingival/oral ☐ In sputum

☐ In vomit ☐ In stool ☐ Vaginal

☐ Line/venepuncture/injection sites ☐ In urine

☐ Other, Specify: _____

Other complication(s) O YES O NO O Unknown

If YES, *Specify*: _____

_____

_____

OUTCOME *(Complete at discharge or death)*

Patient status at outcome: O Discharged (recovered) O Deceased O Transferred to another facility O Fled O Unknown

Date of outcome selected above: *(DD/MM/YYYY)* [____][____]/[____][____]/[__2__][__0__][____][____] O Unknown

If DISCHARGED, Ability to self-care at discharge versus prior to illness: O Same as prior to illness O Worse O Better O Unknown

If TRANSFERRED, Name of new facility: _____

Village/Town: _____

FOLLOW-UP (after discharge – include date of last contact for patients lost to follow-up.)

Date of follow-up: *(DD/MM/YYYY)* [____][____]/[____][____]/[__2__][__0__][____][____]

Status at last contact: O Alive O Deceased

Remaining complications associated with CCHF or CCHF treatment? O YES O NO O Unknown

If YES, Specify: _____

Appendix 4

Study: Pathogenesis of Crimean-Congo Haemorrhagic Fever (CCHF) - interaction of immune response, viral load and clinical course.

INFORMATION SHEET FOR PARTICIPANTS -

25th March 2015. Version 1.3

We are undertaking a research study involving patients with suspected Crimean-Congo Haemorrhagic Fever (CCHF).

You are invited to take part in this study.

Before you decide to take part it is important for you to understand why the research is being done and what it would involve for you. Please take time to read this information carefully. One of our team will go through the information with you. Please ask us if there is anything that is not clear or if you would like more information and time to decide. Your decision is completely voluntary, you can withdraw at any time and the decision you make will not affect your care in any way.

What is the study about?

CCHF is an important infection in Turkey, where the most cases in the world are reported each year. Although it is quite common we still do not understand how the CCHF virus interacts with the human immune system. This research study will gain important information about this disease and may help people in the future.

The main study investigator is Dr Tom Fletcher from the UK who is working with Professor Hakan Leblebicioglu at Ondokus Mayis University Hospital in Samsun.

What will happen if I take part in this study?

We will collect information and blood and urine samples which are in addition to normal medical care. As part of your standard medical care blood samples will be taken every day by the medical team. We would like to take up to 3 small blood tubes at the same time and also collect some urine samples. We would also take another blood and urine sample at around 30 days and a blood sample 1 year from now.

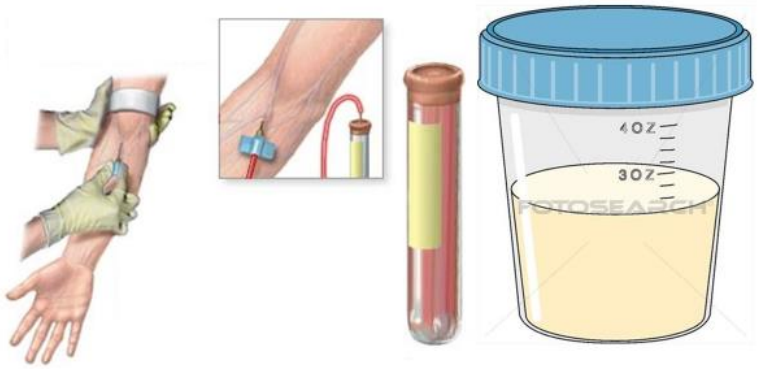

Figure 1: Phlebotomy and urine collection

Will my taking part or not taking part affect my treatment?

No. You should only participate if you want to; choosing not to take part will not disadvantage you in any way with regards to medical care.

What will happen to the samples and information?
We will use the blood samples to look at how the body fights the infection and how the virus interacts with the body. Some of the tests will be done in the UK.

All information about you will be handled in confidence and only the people responsible for your care and for this study will know that you were a part of the study. All information and samples will be labelled only with a number so that neither the samples or test results cannot be directly linked to you personally. Most of the laboratory tests will be completed in Turkey but some specialised tests will be take place in the UK. With your permission, we would also like to store your samples and use them for future ethically approved medical research.

Are there any benefits to taking part in this study?
No. The information gained from this study will not benefit you directly in the management of your illness.

What are the risks of being in the study?
Being a part of this study means that a few samples will be taken that are not needed for normal medical care. There is a small risk of pain or irritation when blood samples are taken but these samples will be planned to be taken at the same time as your routine daily samples.

Can I request that I be withdrawn from the study at any point?
Yes, you can withdraw at any time without giving a reason and without affecting your medical care at all . Any samples that have not already been analysed can be destroyed anytime you request it.

Who is funding the research?
The study is being funded by the Wellcome Trust and the UK Defence Medical services.

What if I have any problems or would like further information about the study?
Please contact the Chief Investigator Dr Tom Fletcher (local mobile, tomfletcher@doctors.org.uk) or the lead Supervisor Prof Hakan Leblebicioglu (unit Number). You can also discuss being involved in the study with an independent doctor who is not part of the research team (Prof Saban Esen tel: 0 362 312 1919 ext 2797).

This study complies, and at all times will comply, with the Declaration of Helsinki [1] as adopted at the 64th WMA General Assemble at Fortaleza, Brazil in October 2013.

_____

[1] World Medical Association Declaration of Helsinki [revised October 2013]. Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Studies. 64th WMA General Assembly, Fortaleza (Brazil).

Appendix 5

CONSENT FORM FOR PARTICIPANTS IN RESEARCH STUDIES

Study: Pathogenesis of Crimean-Congo Haemorrhagic Fever (CCHF) - interaction of immune response, viral load and clinical course

Ministry of Defence Research Ethics Committee Reference: 571/MODREC/14

 The nature, aims and risks of the research have been explained to me. I have read and understood the Information for Participants and understand what is expected of me. All my questions have been answered fully to my satisfaction.

 I understand that if I decide at any time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately without having to give a reason. I also understand that I may be withdrawn from it at any time, and that in neither case will this be held against me or affect my clinical care.

 I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.

 I agree to volunteer as a participant for the study described in the information sheet and give full consent. I understand that this includes the collection of additional blood and urine samples.

 I understand that some specimens taken (blood, urine) may be transported out of the country for analysis.

 This consent is specific to the particular study described in the Information for Participants attached and shall not be taken to imply my consent to participate in any subsequent study or deviation from that detailed here.

Participant's Statement:

I _____

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Participant Information Sheet about the project, and understand what the research study involves. A signed copy of this form will be made available for your personal records

Signature **Date**
or Thumbprint

Impartial witness if patient is illiterate:

Name

Signature **Date**

Next of Kin/Consultees if patient unable to consent:

1. Name	**2. Name**
Signature date	**Signature date**

Investigator's Statement:

I _____

confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the Participant.

Signature **Date**

AUTHORISING SIGNATURES

The information supplied above is to the best of my knowledge and belief accurate. I clearly understand my obligations and the rights of research participants, particularly concerning recruitment of participants and obtaining valid consent.

Signature of Principal Investigator

…… **Date**

Name and contact details of Independent Medical Officer:

Prof Saban Esen Tel: 0362 312 1919 ext 2797.

Name and contact details of Principal Investigator:

Dr Tom Fletcher (#local cell , tomfletcher@doctors.org.uk) or Prof Hakan Leblebicioglu (Unit Number).

Annex 6

Sample Processing Turkey

1. Inspect all samples on reception in the laboratory to ensure no gross blood contamination and the presence of unique patient identifiers on each blood tube.
2. Wearing PPE (Gloves, Lab coat, Face shield) wipe each tube with 0.5% chlorine/vircon solution and allow to dry in the Class 1 BSC
3. All blood samples (except PAXgene tubes) centrifuged at 2000g for 15 minutes at room temperature.
4. Open the centrifuge and inspect the buckets for any leakage.
5. Transfer the sealed buckets to the Class 1 BSC and only open them inside the cabinet wearing PPE.
6. Serum samples: Pipette off serum from the centrifuged tube, and transfer into labelled cryovials (printed labels to indicate sample type, patient details, sample date). 1 x Serum sample tube into 2x1ml aliquots.
7. Plasma/EDTA samples: Pipette plasma from the centrifuged tube, and transferred into labelled cryovials (printed labels to indicate sample type, participant number, sample date). 1 x EDTA sample tube into 2x1ml aliquots.
8. Citrated plasma samples: Separated using plasma 0.5cm above the base of the red cell layer only. The platelet poor plasma (PPP) from is then further processed to obtain platelet free plasma (PFP) or (Double spun plasma) by double spinning the plasma into a clean tube. Plasma is taken off with the plastic Pipette, and transferred into a clean transfer 75 X 12 mm tube, and re centrifuged for 15 minutes at room temperature to obtain platelet free plasma (PFP). The plasma that is left (in the primary blood tube) 0.5mls above the base is put into the single spun cryovial (labelled Single spun plasma). After the second centrifuge PFP samples are then separated into 3 x cryovial tubes in 0.5ml aliquots. The plasma left in the bottom 0.5ml of the second centrifuge is again placed into the SS cryovial.
9. Plasma and serum must be separated within a 3-hour period of being taken. If a delay occurs, then they must be placed at $4^{o}C$, and processed as soon as possible that day.
10. Urine samples: Urine is pipetted directly from the universal container into labelled 1 x 2 ml cryovial (printed labels to indicate sample type, participant number, sample date.) 1 x urine sample tube into 1 x 2ml aliquot.
11. PAXgene tube storage. No sample processing required. Place for 24hrs in -20 freezer then transfer to -80 freezer.